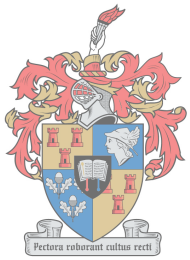
# Neuro- and immunomodulatory effects of *Sceletium tortuosum*

by

Amber Clare Bennett

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# Abstract

Chronic lifestyle stress translates to chronic physiological and psychological conditions. Continual stress-response activation plays a key role in the development of inflammatory diseases, such as diabetes, Alzheimer's disease, anxiety and depression. It is known that inflammatory cytokines produced in the periphery can cross the blood-brain barrier, resulting in release of neurotoxins that can lead to demise of central nervous system integrity. Pro-inflammatory cytokines are also responsible for undesired modulation of serotonin signalling and receptor expression. Since two of the major systems implicated in the aetiology of neurodegeneration and psychological illness are central inflammation and maladaptations in serotonergic signalling, it is important to investigate potential therapeutic targets in this context.

A high-mesembrine *Sceletium tortuosum* extract (Trimesemine™) was shown to possess cytoprotective and mild anti-inflammatory properties in human monocytes, and inhibitory effects on adrenal glucocorticoid synthesis. Additionally, it was recently confirmed that claimed selective serotonin reuptake inhibition (SSRI) properties of *Sceletium tortuosum* are, in fact, secondary to its monoamine-releasing agent (MRA) capacity. A high-Δ7-mesembrenone *Sceletium* extract has also shown promising anti-oxidant capacity and anti-cancer effects. In this thesis, two models were employed to further investigate the therapeutic potential of *Sceletium tortuosum* in maladaptations to chronic stress.

Firstly, the central immunomodulatory effects of two *Sceletium tortuosum* extracts, high-mesembrine extract, Trimesemine™, and high-Δ7-mesembrenone extract, Delta-7, were investigated. Human astrocytes were pre-treated with each extract for 30 minutes, before exposure to *Escherichia coli* lipopolysaccharide for 23.5 hours (in the presence of treatment). Cytotoxicity, mitotoxicity and cytokine responses (basally and in response to inflammatory stimulus) were assessed. Total polyphenol content, antioxidant capacity and selected neural enzyme inhibition capacity were also assessed for both extracts. Trimesemine™ exerted cytoprotective ($p<0.0001$) and anti-inflammatory effects ($p<0.05$). In contrast, Delta-7 exhibited potent antioxidant effects ($p<0.05$), although with relatively higher risk of adverse effects with overdose.

The second model employed was a platelet model of the central serotonergic system, to investigate the effects of high-mesembrine *Sceletium* on serotonin system parameters in anxious and non-anxious individuals. Modulators of the serotonergic system – an SSRI (citalopram) and a mild MRA (Trimesemine™) – were used to investigate the platelet model in anxiety. Isolated platelets from anxious and non-anxious subjects were exposed to a known activator (calcium ionophore A23187) and each modulator for 15 minutes. Cytokine secretion and changes in platelet serotonergic system activity were assessed. Serotonin transporter expression was down-regulated in state anxiety in response to citalopram, validating the platelet model in this context. Basal platelet serotonin levels in individuals exhibiting state/trait anxiety were lower than no-anxiety controls ($p<0.05$), while platelet activation state was increased ($p<0.05$). Trimesemine™ was again confirmed to have anti-inflammatory effect by its modulation of pro- and anti-inflammatory cytokine secretion.

We conclude that both *Sceletium tortuosum* extracts may be employed as either a preventative supplement or complimentary treatment in the context of chronic immune-mediated disease. This study has also highlighted key differences between anxious and non-anxious individuals, and between anxiety type, in terms of serotonergic system parameters and inflammatory responses, suggesting diagnostic potential for the platelet model.

# Uittreksel

Chroniese leefstylstres lei to chroniese fisiologiese en sielkundige toestande. Voortdurende aktivering van die stress respons speel 'n groot rol in die ontwikkeling van inflammatoriese siektes/toestande, soos diabetes, Alzheimer se siekte, angstigheid en depressie. Dit is bekend dat sitokiene wat in die periferie vervaardig word, die bloed-breinskans kan oorsteek en kan lei tot die afskeiding van neurotoksiene wat weer die integriteit van die sentrale senuweestelsel kan aftakel. Pro-inflammatoriese sitokiene is ook vir ongewenste veranderings in reseptor uitdrukking en serotonien seinoordrag verantwoordelik. Aangesien sentrale inflammasie en wanaangepaste serotonien seinoordrag sentraal staan tot die ontwikkelling van neurodegenerasie en psigologiese siektes, is dit bepaald belangrik om moontlike terapeutiese teikens in hierdie konteks te ondersoek.

'n *Sceletium tortuosum* ekstrak met hoë mesembrien inhoud (Trimesemine™) het bewese sitobeskermende en ligte anti-inflammatoriese eienskappe, soos geïllustreer in monosiete, asook beperkende effekte op bynier glukokortikoïed sintese. Onlangse navorsing het ook bewys dat die selektiewe serotonien heropname inhibisie (SSRI) effek wat algemeen aanvaar word vir die ekstrak, eintlik sekondêr is tot 'n mono-amien vrystellings (MRA) funksie. In voorlopige studies het 'n *Sceletium tortuosum* ekstrak met hoë Δ7-mesembrenoon inhoud ook belowende anti-oksidant en teenkankereffekte getoon. In hierdie tesis word twee modelle gebruik om die terapeutiese potensiaal van *Sceletium tortuosum* in die konteks van wanaanpassings as gevolg van chroniese stres, verder toe te lig.

Eerstens is die sentrale immuun-modulerende effekte van twee *Sceletium tortuosum* ekstrakte – een met hoë mesembrien inhoud, Trimesemine™, en een met hoë Δ7-mesembrenoon inhoud, Delta-7, ondersoek. Menslike astrosiete is vir 30 minute voorafbehandel met elke ekstrak, waarna dit vir 23.5 uur aan *Escherichia coli* lipopolisakkaried blootgestel is (in die teenwoordigheid van ekstrak). Sitotoksisiteit, mitotoksisiteit en sitokienreaksies (beide basal en in reaksie op die inflammatoriese stimulus) is bepaal. Totale polifenoolinhoud, anti-oksidant kapasiteit en inhibisie van relevante neurale ensieme is ook vir beide ekstrakte bepaal. Trimesemine™ het sitobeskermende ($p<0.0001$) en anti-inflammatoriese ($p<0.05$) effekte getoon. In kontras daarmee, het Delta-7 sterk anti-oksidant effekte getoon ($p<0.05$), alhoewel dit met 'n relatiewe hoër risiko vir ongewenste effekte van oordosering verbind kan word.

Die tweede model was 'n plaatjie-model van die sentrale serotonergiese sisteem, waarmee die effek van hoë-mesembrien *Sceletium* op parameters van die serotonien sisteem in beide angstige en nie-angstige individue bepaal is. Moduleerders van die serotonergiese sisteem – 'n SSRI (citalopram) en 'n ligte MRA (Trimesemine™) – is aangewend om die plaatjiemodel in die konteks van angstigheid te toets. Plaatjies geïsoleer uit angstige en nie-angstige skenkers is vir 15 minute saam met 'n bekende aktiveerder (calcium ionophore A23187) aan elke moduleerder blootgestel. Sitokien afskeiding en veranderinge in plaatjie parameters wat die serotonien sisteemaktiwiteit aandui, is bepaal. Serotonien vervoerreseptor (SERT) uitdrukking is afgereguleer na citalopram behandeling, in toestand-angstigheid, wat die plaatjie-model bekragtig. Basale plaatjie serotonienvlakke was laer in individue met beide toestand- en eienskap-angstigheid, in vergelyking met nie-angstige kontroles ($p<0.05$), terwyl plaatjie aktiveringstatus verhoog was

($p<0.05$). Anti-inflammatoriese effek van Trimesemine™ is weer bevestig met die modulering van pro- en anti-inflammatoriese sitokiene.

Ten slotte bevind ons dat beide *Sceletium tortuosum* ekstrakte as voorkomende of komplimentêre behandeling in die konteks van chroniese siekte gebruik kan word. Hierdie studie lig ook belangrike verskille tussen angstige en nie-angstige individue, asoos tipes angstigheid, uit, in terme van parameters van die serotonien sisteem en inflammatoriese reaksies, wat diagnostiese potensiaal van die plaatjie-model mag aandui.

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# Research Outputs

## Conferences

Physiological Society of Southern Africa (PSSA) Conference (August 2016). River Club, Cape Town, South Africa. Poster Presentation: "*Sceletium tortuosum* and Depression: Mechanisms Elucidated".

Conferences on Trans-Pyrenean Investigations in Obesity and Diabetes (CTPIOD): 14th French-Spanish Meeting, 2017. San Jorge University, Villanueva de Gállego, Zaragoza, Spain. Oral Presentation: "*Sceletium tortuosum* alkaloids may delay chronic disease progression via anti-inflammatory action".

## Articles

Bennett, A., Lopez, V., van Camp, A., Smith, C., 2016. *Sceletium tortuosum* and depression: mechanisms elucidated. Planta Med. 81. https://doi.org/10.1055/s-0036-1596865.

Bennett, A.C., Smith, C. Immunomodulatory effects of *Sceletium* extract (Trimesemine) elucidated *in vitro*: Implications for chronic disease. Journal of Ethnopharmacology (minor revisions resubmitted).

Bennett, A., Lopez, V., van Camp, A., Smith, C. *Sceletium tortuosum* may delay chronic disease progression via alkaloid dependant antioxidant or anti-inflammatory action. Invited paper submitted to Journal of Physiology and Biochemistry.

Bennett, A.C., Smith, C. Diagnosis and drug discovery in acute and chronic anxiety: illustrated relevance of the platelet serotonergic system. Submitted to Journal of Affective Disorders.

# Contents

# List of Acronyms & Abbreviations

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine |
| AChE | Acetylcholinesterase |
| ADP | Adenosine diphosphate |
| APTT | Activated partial thromboplastin time |
| ATP | Adenosine triphosphate |
| BBB | Blood-brain barrier |
| CD41a | Cluster of Differentiation 41a |
| CD63 | Cluster of Differentiation 63 |
| CNS | Central nervous system |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DPBS | Dulbecco's phosphate buffered saline |
| DPPH | 2,2-diphenyl-1-picryl-hydrazyl |
| FBS | Foetal bovine serum |
| FMO | Fluorescence minus one |
| FSC | Forward scatter |
| GC(s) | Glucocorticoid(s) |
| GTP-CH1 | Guanosine triphosphate cyclohydrolase-1 |
| HBSS | Hank's buffered salt solution |
| HPA axis | Hypothalamic-pituitary-adrenal axis |
| HPLC | High-performance liquid chromatography |
| IgG | Immunoglobulin-G |
| IL-1, -4, -6 etc. | Interleukin-1, -4, -6 etc. |
| IL-1β | Interleukin-1beta |
| INR | International normalized ratio |
| LAMP-2 | Lysosome associated membrane protein-2 |
| LPS | Lipopolysaccharide |
| MCP-1 | Monocyte chemotactic protein-1 |
| MFI | Mean fluorescent intensity |
| MR | Mineralocorticoid receptor |

| | |
|---|---|
| MRA | Monoamine-releasing agent |
| NAP2 | Neutrophil-activating peptide-2 |
| PBS | Phosphate buffered saline |
| PF-4 | Platelet factor-4 |
| PI | Propidium iodide |
| PMT | Photomultiplier tubes |
| PPP | Platelet-poor plasma |
| PRP | Platelet-rich plasma |
| ROS | Reactive oxygen species |
| RPM | Revolutions per minute |
| SAM axis | Sympathetic–adrenal-medullary axis |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SERT | Serotonin transporter |
| SSC | Side scatter |
| SSRI | Selective serotonin reuptake inhibitor |
| STAI | State-Trait Anxiety Index |
| T2D | Type II diabetes |
| T3D | Type III diabetes |
| TNF-α | Tumour necrosis factor-alpha |
| TPH | Tryptophan hydroxylase |
| TRI | Trimesemine |
| VMAT-2 | Vesicular monoamine transporter-2 |
| XTT | 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium- 5-carboxanilide |

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# Chapter 1 – Introduction

Despite stress having largely negative associations, it is an ever-present aspect of daily life, acting as a driving force for some, but as a burden for most individuals. A cohesive description of stress indicates that it consists of a stressor (in any form) that prompts a reaction in the brain and activates stress-response systems within the body (McTeague and Lang, 2012; Morag *et al*., 1999; Padgett and Glaser, 2003).

Continual, unrelenting lifestyle stress resulting from everyday stressors that are poorly managed, or from traumatic events, has been found to translate to chronic psychological conditions (Ross *et al*., 2017). It is therefore understandable that the prevalence of common mental health disorders is on the rise. From 1990 to 2013 alone, the number of reported cases of anxiety and/or depression increased by ±50%. Almost 10% of the global population are afflicted, with mental health disorders accounting for 30% of the world's non-fatal disease burden (World Health Organisation, 2016).

Besides the obvious morbidity that accompanies chronic anxiety and depression, continual stress-response activation plays a key role in the development of inflammatory lifestyle diseases such as type 2 diabetes (T2D), cardiovascular diseases, autoimmune diseases (O'Donovan *et al*., 2014), allergies, Alzheimer's disease and pain (Himmerich *et al*., 2008; Kiecolt-Glaser *et al*., 2015; Knol *et al*., 2006; Postal and Appenzeller, 2015). Likewise, the presence of these conditions is also characterised by an elevated risk for development of mental health disorders (Dantzer *et al*., 2008; Walker *et al*., 2014), indicating a distinct, self-perpetuating relationship between central and peripheral maladaptations to physiological and psychological stress.

It is also commonly known that an extended process is often required to arrive at suitable dose and type of anti-depressant or anxiolytic drugs for any individual because of suboptimal diagnostic practise, since observing pathological changes within the central nervous system (CNS) remains significantly more challenging than for peripheral organs. This is mainly due to anatomic restrictions, as well as the relative risks involved in acquiring brain samples for further investigation.

Current therapeutic options for mental health disorders mainly focus on modulation of the serotonergic system, either through increasing serotonin availability in synapses within the CNS or prolonging its signalling time by preventing reuptake into the presynaptic neuron. Modulators of this system have also been found to possess anti-inflammatory properties (Abdel-Salam *et al*., 2004; Sawynok *et al*., 2001), substantiating the distinct link between the serotonergic- and the inflammatory immune systems. However, current drugs employed for the treatment of anxiety and/or depression are known to elicit adverse effects such as drowsiness, nausea, headaches, and reduced libido.

Recently, interest in the herbal supplement, *Sceletium tortuosum*, has been on the rise, as it may be beneficial in reducing the symptoms of anxiety and depression (Shikanga *et al*., 2012; Smith and Weyrich, 2011), as well as limit the inflammatory response and glucocorticoid (GC) synthesis (Swart and Smith, 2016). These findings

highlight the key role that *Sceletium tortuosum* extract supplementation – alone or in conjunction with other commercial anxiolytic/antidepressant drugs – may play in the treatment of non-communicable inflammatory disorders, However, research data from physiologically-relevant models is still scant.

The following chapter will introduce the complexity of the chronic stress-chronic inflammation-chronic disease relationship, with a specific focus on its contribution to the pathophysiology of inflammatory mental health disorders, with specific focus on neurodegeneration and anxiety. A potentially novel model in the context of the diagnosis and therapeutic monitoring of anxiety will then be explored, drawing parallels to central neurons most affected in this condition. Lastly, current pharmaceuticals employed in the treatment of anxiety/depression disorders will be discussed, followed by a reference to *Sceletium tortuosum* as a potential treatment, given its similar pharmacological actions to popular commercial drugs in this setting, and promising immunomodulatory properties.

This thesis is divided into two major bodies of work, based on manuscripts that have been submitted for publication in two different journals (*Journal of Physiology and Biochemistry* and *Journal of Affective Disorders*) and are currently under review for publication. Thus, general background pertaining to both bodies of work will be covered in Chapter 2, but more detailed literature most specific to the two different experiments will introduce each respective chapter.

# Chapter 2 – Literature Review

The sheer size of the recent body of literature on chronic inflammatory illnesses testifies to the increasing incidence of these conditions and the relative burden its places on not only personal health, but also the health care system and society in general. From my review of the literature, it became evident that most non-communicable inflammatory diseases have interlinked aetiologies. Inflammation and poor lifestyle habits are central to the progression from adaptation to maladaptation to various initial triggers. A complexity in this context is that various factors interlink, forming a tangle of self-propagating cycles (presented in Figure 2.1), so that once initiated, it is very difficult to discern specific primary causes to target therapeutically.

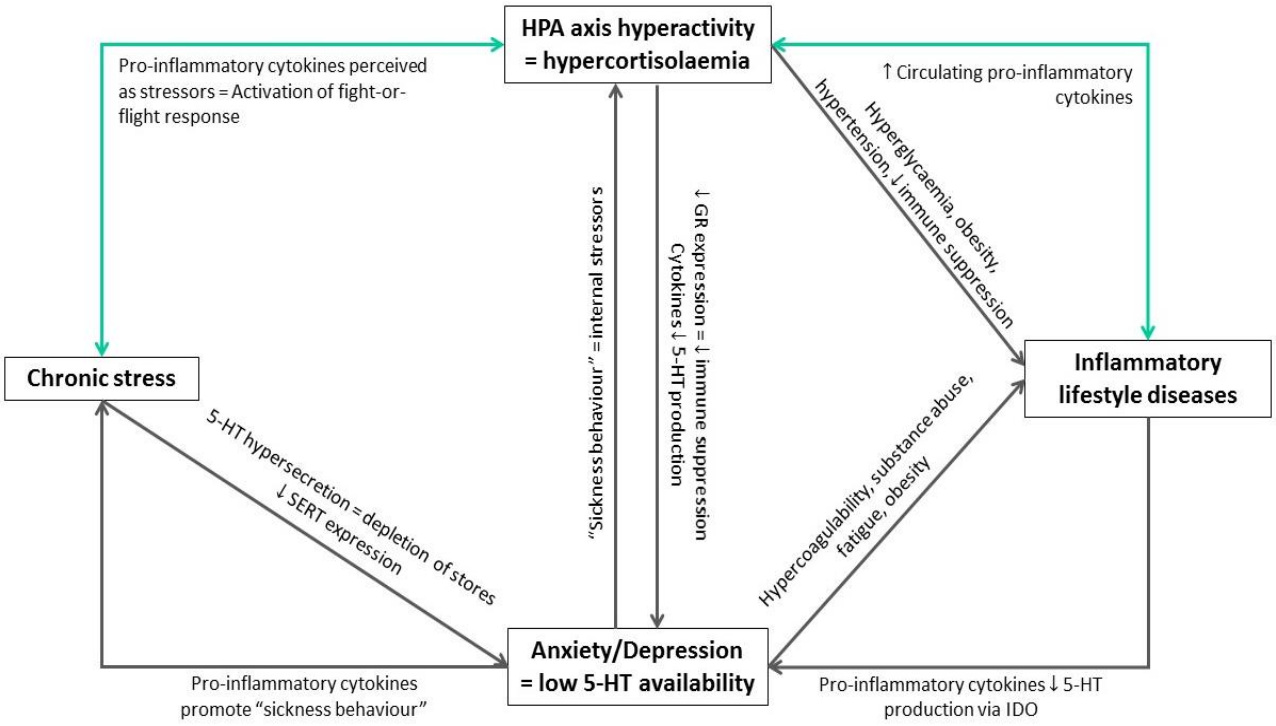



*Figure 2.1: A summary of the complex relationship between chronic stress, inflammation and the development of chronic central and peripheral disease*

For this thesis, I have narrowed the scope to focus on physiological systems associated with the modern stressful lifestyle. In particular, I will focus on the serotonergic and inflammatory immune systems in the context of anxiety/depression. However, cross-links to other chronic diseases cannot be disregarded – for example, obesity has been described as a risk factor for mental health disorders (Anstey *et al*., 2011; Silić *et al*., 2012). Thus, I will attempt to interpret my experimental data relevant to not only anxiety/depression, but also other conditions as applicable.

In the next few pages, I will provide a brief description of the physiology of anxiety and depression. This will include most common causes of these conditions, as well as the major role players involved in stress-immune

cross-talk. I then move on to discuss how these systems may be modulated for therapeutic effect, or as preventative measure. Given the obvious difficulty of investigating potential modulation of the serotonergic system, I argue the potential of using peripheral blood platelets as model with which to simulate the serotonergic system. Lastly, I present available literature on a natural product which hold potential as natural medicine in this context.

## 2.1 The Physiology of Anxiety and Depression

Anxiety disorders are consistently reported to be the most prevalent of mental health disorders, with an estimated 12-month prevalence of 11% and a lifetime prevalence of 16% (Kessler *et al*., 2011). Additionally, depression affects approximately 4% of the total world population (World Health Organisation, 2017). The significant co-morbidity of anxiety and depression accounts for up to 70% of patients with one disorder experiencing the other during their lifetime (Gorman, 1996).

In my opinion, the maladaptations of the body's control systems (endocrine, immune and neural) are present – but not reliably monitored or detected – long before clinical manifestation of the physical symptoms of chronic disease. In support of this, many of these early-onset maladaptations are important predictors of subsequent onset of a number of physical morbidities (Knol *et al*., 2006; Ormel *et al*., 2007).

Following an extensive review of the literature, it was concluded that although anxiety and depression are two distinctly different conditions, once clinical pathology has set in, the two cannot be reliably separated, as one is rarely observed without the other. This of course adds complexity to integrative interpretation of the literature on this topic. Thus, although anxiety is the context for this thesis, I will present literature covering both anxiety and depression, in terms of their maladaptations and their contributions to inflammation and chronic disease development.

### 2.1.1 Pathophysiology in anxiety

The concept of fear can be defined as a general response to unknown threats or to internal conflict. Anxiety is a more exacerbated form of fear, combining subjective emotional distress with physiological stress-response activation and fear behaviour (McTeague and Lang, 2012; Steimer, 2002). Since the main role of fear is to trigger adaptive responses to maintain homeostasis, it is understandable that anxiety stems from exaggerated fear-mediating mechanisms that have been developed to protect us from distant threats, rather than immediate ones. Despite anxiety being a natural reaction, it can develop into a pathological inability to cope with stressful events, characterised by exaggerated emotional responses to stimuli that are perceived as innocuous or only mildly threatening by others (McTeague and Lang, 2012).

Three major vulnerability concepts for anxiety development in humans have been defined: (i) generalised biological vulnerability of genetic origin, (ii) generalised psychological vulnerability, due to early life experiences, and (iii) specific psychological vulnerability, resulting from particular traumatic life events (Behar

*et al*., 2009). While the first two concepts are mostly implicated in the development of generalized anxiety disorders, the latter is significant in the development of specific anxiety disorders such as obsessive-compulsive disorder, social phobia and panic disorder (Steimer, 2002).

Normal arousal in the fear response results from increased excitability of serotonergic, GABA-ergic and noradrenergic neurons (Roth, 2005), in preparation for physical reactions to a perceived threat. An increase in sympathetic signalling via the sympathetic-adrenal-medullary (SAM) axis results in preparation for the fight-or-flight response, and hormones released through the hypothalamic-pituitary-adrenal (HPA) axis mediate energy store release (more detail on this in the following section).

Sustained arousal through chronic anxiety can deplete neurotransmitters, affect receptor expression and cause chronic stress-response signalling, resulting in the development of emotional and somatic depressive symptoms (Charney and Drevets, 2002; Gorman, 1996; Steimer, 2002).

### 2.1.2 Pathophysiology in depression

The core, overlapping symptoms of anxiety and clinical depression include irritability and agitation, concentration difficulties, and disrupted sleeping and eating patterns (Gorman, 1996; Nestler *et al*., 2002). Brain areas mostly implicated in these mental disorders are those involved in emotion processing, emotional memory formation and retrieval, including the prefrontal cortex, subgenual cingulate cortex, subcortical hippocampus and the amygdala (Krishnan and Nestler, 2008; Ressler and Mayberg, 2007).

Biological maladaptations common to both anxiety and depression include altered serotonin (5-hydroxytryptamine, 5-HT) transmission and HPA axis dysfunction (Zangrossi and Graeff, 2014). 5-HT is a monoamine that regulates a range of functions in both the central nervous system (CNS) and in the peripheral compartment (Berger *et al*., 2009; Jacobs and Azmitia, 1992; Ressler and Mayberg, 2007).

In contrast to 5-HT hypersecretion in anxiety, depression results from ineffective post-synaptic 5-HT transmission. $5\text{-HT}_{1A}$ receptors, the main inhibitory 5-HT receptor subtype responsible for limiting the effects of serotonergic signalling within the CNS and periphery, increase in response to elevations in circulating 5-HT. Ultimately, this results in a decrease in 5-HT transmission (Fajardo *et al*., 2003) - and depressive symptomology - since 5-HT regulates a broad range of behaviours and processes, including mood, appetite, memory, aggression and sleep.

Since the development of anxiety and depression is largely attributed to maladaptation of the major stress-response systems, which are subsequently further stimulated by the bi-products of these mental health disorders, one can understand the unavoidable overlap in literature on anxiety and depression research. Since chronic stress is a major contributor to the overstimulation of physiological stress-response mechanisms, it is important to cover its role in this context in more detail.

### 2.1.3 How does chronic stress cause anxiety/depression?

This section provides basic information on the normal functioning and related role-players of three chosen major mechanisms that link the stress and immune systems, before discussing these maladaptive processes in more detail in the context of anxiety/depression.

#### *2.1.3.1 Cytokines*

All inflammatory cytokines can be secreted under various conditions, and these molecules can stimulate a range of effector functions of both innate and adaptive immunity (Medzhitov and Janeway, 1997). The net effect of any human cytokine is dependent on the timing of cytokine release, the environment in which it exerts its effect, the presence of antagonist or synergistic elements, and the tissue responsiveness (Barak *et al*., 2002; Blanco *et al*., 2008; Dinarello, 1997).

Inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) are known as multi-potential mediators of the immune system, as they have a wide range of biological activities. Depending on their local concentration, they can have favourable or unfavourable effects on the host inflammatory response (Barak *et al*., 2002).

Dysregulation of the functional action of the immune system in chronically stressed patients is a phenomenon that has been confirmed many times (Anisman and Merali, 2002; Iwata *et al*., 2013; Kiecolt-Glaser *et al*., 2015; Miller *et al*., 2009; Schiepers *et al*., 2005). In 1999, Maes proposed the "IRS model of major depression", which explains that mental health disorders may be linked to chronic stimulation of the inflammatory response system. Per this model, anxiety and depression are classified as psycho-neuro-immunological disorders, in which the continued low-level release of pro-inflammatory cytokines by activated immune cells is responsible for the range of maladaptive behaviours and neuroendocrine changes associated with this condition. This assumption has been consistently expressed as a range of hypotheses, all with common backbone. A summary of these can be seen in Table 2.1.

Table 2.1: A summary of the major inflammation-mental health disorder hypotheses

| Hypothesis | Comments | References |
|---|---|---|
| The monocyte-T lymphocyte hypothesis | Activated macrophages contribute to the pathophysiology of depression by direct IL-1β stimulation on the HPA-axis (see HPA-axis hyperactivity hypothesis). | (Leonard, 2001; Maes, 1995; Maes *et al*., 1998) |

| | | |
|---|---|---|
| IRS model of depression | Major depression results from products of a dysregulated inflammatory response system. | (Dowlati *et al*., 2010; Maes, 1999) |
| HPA-axis hyperactivity hypothesis | HPA axis hyperactivity results in decreased GR expression; therefore, inflammatory cytokines are persistently secreted, interfering with 5-HT turnover and ultimately development of depressive symptoms. | (Dantzer *et al*., 2008; Flandreau *et al*., 2012) |
| The cytokines hypothesis of depression | Chronic peripheral immune challenge can induce neuroinflammation, resulting in changes in neurotransmitter metabolism which may lead to 5-HT deficiency.<br><br>Depression may be a sickness behaviour result from chronic immune cell activation. | (Dantzer, 2004; Haase and Brown, 2015) |
| The psycho-immunological hypothesis | Mood disorders are attributed to an abnormal proneness to systemic inflammation (specifically those suffering from inflammatory lifestyle diseases, and aged populations) | (Dantzer *et al*., 2008) |
| The monoamine hypothesis of depression | Chronic circulating pro-inflammatory cytokines reduce tryptophan availability in the CNS and interfere with 5-Ht production | (Capuron *et al*., 2003; Leonard, 2014; Maes *et al*., 2011) |

Significant similarities exist between cytokine-induced sickness behaviour and anxiety/depression. Both conditions are characterised by physical and social withdrawal, pain, fatigue and low mood (Dantzer, 2004;

Dantzer *et al*., 2008). Major pro-inflammatory maladaptations exist in patients with mental health disorders in comparison to healthy controls (Miller *et al*., 2009; Valkanova *et al*., 2013). This supports the idea of an interconnectedness of anxiety, depression and inflammation. A summary of the major cytokines assessed in anxiety/depression research is provided in Table 2.2.

Table 2.2: Common cytokines/chemokines used as biomarkers in chronic stress and anxiety/depression research

| Cytokine/chemokine | Comments | References |
|---|---|---|
| C-reactive protein | Elevated levels in blood of depressed patients. | (Leonard, 2014) |
| IL-10 | Serum levels are reduced in patients with depression. Subjects with stress-induced anxiety showed significantly lower serum IL-10. | (Berk *et al*., 2013; Leonard, 2014, 2001; Maes *et al*., 1998) |
| IL-1β | Systemic administration of lipopolysaccharide (LPS) in rats induces IL-1β secretion in the CNS, resulting in sickness behaviour. Plasma IL-1β levels were significantly elevated ($p<0.05$) in depressed patients. | (Anisman and Merali, 2002; Dahl *et al*., 2014; Dantzer *et al*., 2008; Lopresti *et al*., 2014) |
| IL-6 | Peripheral marker of chronic stress-related inflammation. Associated with an increased risk of development of depressive symptoms. Dysregulated sleep patterns in anxiety/depression associated with increased IL-6 levels. SSRI treatment causes a decrease in IL-6 and TNF-α levels. | (Frommberger *et al*., 1997; Lopresti *et al*., 2014; Miller *et al*., 2009; Valkanova *et al*., 2013) |
| IL-8 | Plasma IL-8 levels were significantly elevated ($p<0.05$) in depressed patients. | (Dahl *et al*., 2014) |
| MCP-1 | Elevated levels in the cerebral-spinal fluid in patients with mood and anxiety disorders. | (Mackay, 2015) |
| Platelet factor-4 | Increased platelet secretion of platelet factor-4 (PF-4) in subjects performing mental stress tasks | (Koudouovoh-Tripp and Sperner-Unterweger, 2012) |
| TNF-α | Under normal conditions: important for enhancing neurogenesis and providing trophic support. Elevated serum concentrations in depressed patients compared to controls. | (Dahl *et al*., 2014; Dowlati *et al*., 2010; Himmerich *et al*., 2008; Miller *et al*., 2009; Postal and Appenzeller, 2015) |

| | | |
|---|---|---|
| | Elevated levels in the CNS contribute to neurodegeneration, oxidative stress and apoptosis of astrocytes. | |

The benefit of having an established panel of cytokine biomarkers for mental health disorder characterisation is that their presence or absence can be used to indicate probability of onset or estimate current disease status. In addition, biomarkers can be used to classify disorder severity based on the maladaptations present. Most importantly, they may be useful indicators of therapeutic efficacy and provide insight into a patient's personal responses to treatment options.

Excessive cytokine production in any setting can cause damage (Lu *et al*., 2010; Streit *et al*., 2004). Therefore, limitation of inflammatory cytokine production – in circulation, but also specifically locally at tissue level – should be beneficial for prevention of chronic systemic inflammation, and consequent development of inflammation-related illness (Lu *et al*., 2010; Rothwell and Hopkins, 1995; Streit *et al*., 2004).

In summary, the immune system is regulated, to a significant extent, by both the central and peripheral nervous systems, with bidirectional communication occurring between these two systems, in conjunction with the endocrine system (Basu and Dasgupta, 2000; Padgett and Glaser, 2003). Therefore, it is important to next discuss the role of the HPA axis and its chemical constituents in the context of chronic stress and anxiety/depression development.

#### *2.1.3.2 Glucocorticoids*

The HPA axis is central in stress response mediation. During times of stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), which acts on the anterior portion of the pituitary gland. This stimulates release of adrenocorticotropic hormone (ACTH), resulting in increased GC production and secretion by the adrenal cortex of the adrenal glands. In acute stress, elevated GC levels regulate a number of physiological processes that are vital for mediation of "fight-or-flight" responses (Elenkov, 2008; Padgett and Glaser, 2003), such as metabolism, immune function, cardiovascular function, and cognition (Oakley and Cidlowski, 2013).

The actions of GCs are mediated by binding to the widely-distributed glucocorticoid receptor (GR). Negative feedback mechanisms regulate HPA axis activity via GC binding to mineralocorticoid receptors (MRs) localised in the hippocampus, and GRs. GR binding terminates physiological responses to the acute stressor, facilitating restoration of homeostatic balance (Haddad *et al*., 2002). In chronic stress, adaptations occur in response to continual GC release, with downregulation of GR expression, resulting in increased activity in the MR system; this results in pathological hyperactivation of the HPA axis (*Figure 2.2*).

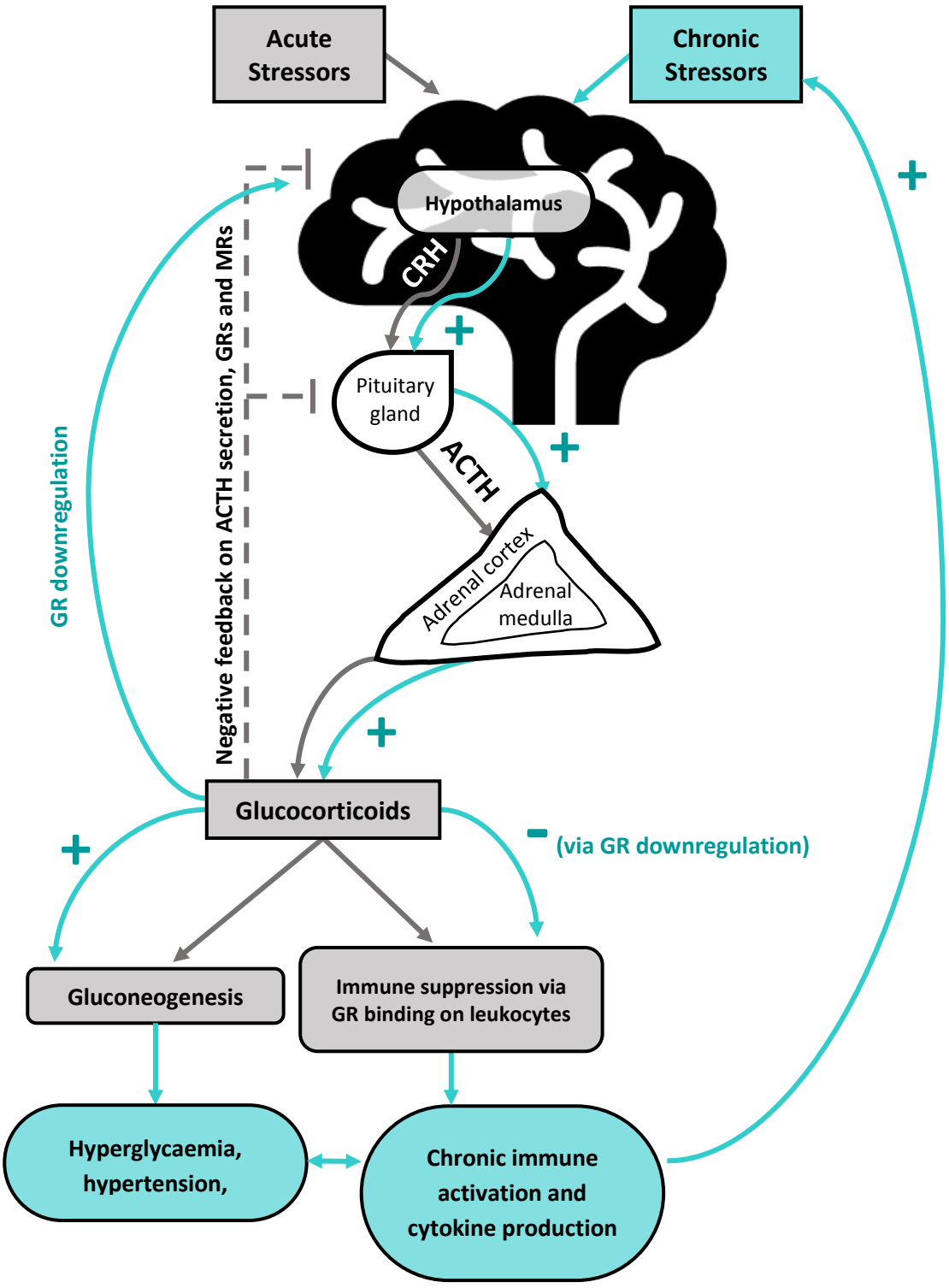



*Figure 2.2: The hypothalamic-pituitary-adrenal axis physiology in acute stress, and maladaptations in response to chronic stress. A self-perpetuating cycle exists between chronic stress and HPA hyperactivity, through the products of hypercortisolaemia (redrawn and modified from Elenkov (2008), Flandreau et al. (2012), and Stokes (1995))*

Downregulation of GR expression in circulating leukocytes results in an inability for GCs to limit the inflammatory response. Since an immune challenge that threatens homeostasis can be regarded as a stressor, the products of an activated immune system can further stimulate CRH secretion during

inflammatory stress. Continuous inflammatory insults result in hypercortisolaemia, exacerbating systemic GR expression downregulation in chronic stress.

Constantly elevated circulating GC levels means that the physiological stress response is always activated. In a normal "fight-or-flight" situation, gluconeogenesis occurs to provide energy for increased metabolism and muscle functionality, the results of which are an increase in blood-glucose levels, heart rate and blood pressure. Continual gluconeogenesis causes chronic hyperglycaemia, hypertension, cardiovascular complications such as arrhythmia and hypercoagulation, and insomnia (MacLaughlin *et al*., 2011; Oakley and Cidlowski, 2013). These conditions are precursors to the development of lifestyle illnesses such as neurodegeneration and T2D which further contribute to systemic inflammation (Elenkov, 2008), with pro-inflammatory cytokines feeding back into the HPA axis.

Having briefly reviewed the general literature on role players involved in major stress-immune cross-talk, the next section will focus more specifically on the role of the serotonergic system and its links to the inflammatory immune system in the setting of chronic stress.

#### *2.1.3.3 Serotonin*

Serotonergic dysfunction is a central focus in the pathophysiology, as well as the treatment of, anxiety and depression (Sorgdrager *et al*., 2017). Exposure to chronic stressors results in reduced 5-HT availability in the prefrontal cortex, reflecting a state in which synthesis of 5-HT is outpaced by release (Charney and Drevets, 2002). These effects are also observed in circulating blood platelets, where 5-HT release occurs more rapidly than 5-HT reuptake, resulting in haematological and immunological disorders that will be discussed in subsequent sections.

The serotonin transporter (SERT) plays a fundamental role in the maintenance of serotonergic function, by regulating 5-HT uptake into cells (Hoirisch-Clapauch *et al*., 2014; Velenovská and Fišar, 2007). SERT is found in cells in both the peripheral and central compartments, mainly those of the gastrointestinal tract, platelets and serotonergic neurons (Dürk *et al*., 2005; Hoirisch-Clapauch *et al*., 2014; Scharinger *et al*., 2014).

SERT is a membrane integral glycoprotein, and a member of the SLC6 gene family of $Na^+/Cl^-$dependant transporter proteins (Hoirisch-Clapauch *et al*., 2014). The 5-HT uptake process involves its binding to the recognition site on SERT, and its transport across the membrane in conjunction with a $Na^+$ ion. The second step involves the translocation of $K^+$ across the membrane to the exterior of the cell. The requirement for $K^+$ is unique to SERT within the SLC6 family (Jedlitschky *et al*., 2012).

Apart from transmission, the synthesis of neurotransmitters in the CNS is another important consideration, as their inhibited or upregulated synthesis may also have undesired effects. 5-HT synthesis is dependent on the availability of its precursor amino acid, tryptophan. Two major pathways metabolise tryptophan: the

kynurenine pathway, regulated by the enzyme indoleamine 2,3-dioxygenase (IDO), and the 5-HT pathway, which is initiated by the tryptophan 5-monooxygenase enzyme (Dantzer *et al*., 2008).

In this context, inflammation was shown to have a role in pathology. IDO enzymes in macrophages and T-lymphocytes, assist in degradation of tryptophan to generate kynurenines and kynuramines. These compounds function in negative feedback loops to modulate 5-HT-mediated inflammation, as well as pro-inflammatory molecules (Arreola *et al*., 2015; Greeson *et al*., 2015). Therefore, the constant presence of circulating pro-inflammatory cytokines reduces tryptophan availability in the CNS, and ultimately 5-HT availability (Figure 2.3).

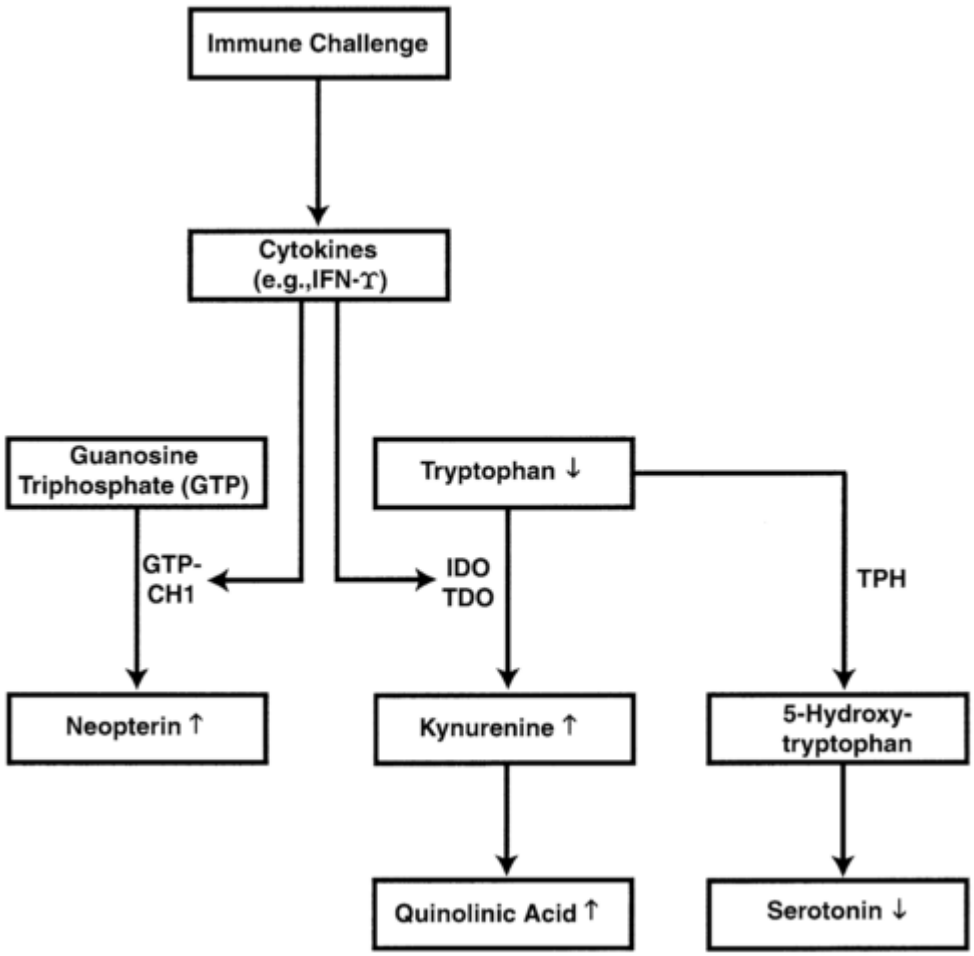



*Figure 2.3: Cytokines stimulate catabolism of tryptophan into kynurenine and quinolinic acid via induction of IDO, resulting in decreased availability of tryptophan for 5-HT synthesis via tryptophan hydroxylase (TPH). Cytokines also induce neopterin synthesis by activated macrophages via guanosine triphosphate cyclohyrdolase-1 (GTP-CH1) (Capuron et al., 2003)*

The chronic shunting of tryptophan into the kynurenine pathway is associated with the aetiology of anxiety and depressive disorders, as formulated in the monoamine hypothesis of depression (Miura *et al*., 2008; Schiepers *et al*., 2005; Sorgdrager *et al*., 2017).

The literature reviewed up to this point provides clear evidence of the neuro-immune crosstalk, as well as its maladaptation, involved in progression towards chronic disease. It is clear that in the context of anxiety, inflammation and compromised 5-HT availability are central to this maladaptations, and thus clear therapeutic targets. However, before treatment options can be considered or evaluated, I would like to briefly discuss a novel potential model with which to investigate modulations of neuro- and immune role players.

Blood platelets are known to secrete 5-HT, although this action is usually assessed as an indicator of platelet activation in hypercoagulability studies. However, more relevant to the context of the current thesis, the next section discusses the feasibility of using platelets as a model for the serotonergic system in the context of depression/anxiety research.

## 2.2 Platelets to model the central serotonergic system in anxiety?

Available techniques to study brain function depend on neuroimaging techniques – which rely on indirect markers of activation (Oathes *et al*., 2015) – and post-mortem examination, which presents with major limitations. Even though these techniques have provided valuable insight into affected brain regions in anxiety and depression, they do not elucidate the array of mechanisms involved in the development of these chronic mental disorders (Ressler and Mayberg, 2007). Given the close links between the immune and serotonergic system, it may be possible to employ more accessible samples to model the serotonergic system, both for diagnostic and therapeutic monitoring assessments.

As previously mentioned, 5-HT is best known as a neurotransmitter involved in mood-related disorders. However, it is also responsible for mediating platelet aggregation via the 5-HT receptor 5-$HT_{2A}$. In addition, in the context of haemostasis, it acts on endothelial cell receptors and elicits production of nitric oxide specifically in undamaged cells, which controls the extent of coagulation by vasodilation and inhibition of platelet aggregation, so that clot formation is restricted to damaged areas only (Jedlitschky *et al*., 2012).

More directly relevant to the thesis topic of immune-stress cross-talk, platelet 5-HT has been shown to modulate the inflammatory response (Mumford *et al*., 2015), the mechanisms of which will be discussed later in this section. Therefore, it is of interest to understand the role of the platelet in 5-HT turnover.

Platelets are small cell fragments of megakaryocytes, found in circulation at a density of 150-450 x $10^9$/litre of blood. Although they are anucleate, they perform a great number of functions, mainly involved in the maintenance of vascular integrity through sensing injury to blood vessel endothelium, accumulating at a site of damage, and subsequently causing blood clotting to close the wound and promote tissue repair (Jedlitschky *et al*., 2012; Scull *et al*., 2010).

In addition to their secretory products, platelets also possess several immune receptors on their surface, including toll-like receptors and immunoglobulin receptors (Duerschmied *et al*., 2014; Gawaz *et al*., 2005; Starossom *et al*., 2015). The proposed mechanism by which platelets achieve immunomodulation is through their direct interaction with cells of the innate immune system (Gawaz *et al*., 2005; Speth *et al*., 2013).

Upon activation, platelets undergo a series of morphological changes, including shape change and membrane budding, granular content release, adhesion and aggregation. Measures of platelet activation utilise these changes, which are assessed either directly (scanning electron microscopy, flow cytometry) or indirectly by measuring metabolites of activated platelets in plasma or urine (ELISA) (Choudhury *et al*., 2007).

### 2.2.1 Platelet granules

Platelets contain mRNA as well as the full translation machinery required for protein synthesis. These proteins are then stored in lysosomal granules (≤3 per platelet), dense bodies (or δ-granules) (3-8 per platelet) and α-granules (50-80 per platelet) (Duerschmied *et al*., 2014; Speth *et al*., 2013). Platelets maintain normal haemostasis by releasing granule contents at sites of vascular damage (Ambrosio *et al*., 2012; Jedlitschky *et al*., 2012; Jonnalagadda *et al*., 2012; Smyth *et al*., 2009). A summary of the main constituents of each granule type is presented in Table 2.3.

Table 2.3: Platelet granules and corresponding contents

| α-granules | δ-granules/dense granules | Lysosomes |
|---|---|---|
| Platelet factor-4 | ADP | β-hexosaminidase |
| Multimerin | ATP | LAMP-2 |
| Adhesive glycoproteins | 5-HT | |
| Fibrinogen | Calcium | |
| von Willebrand factor | GDP | |
| von Willebrand pro-peptide | Pyrophosphate | |
| Thrombospondin | Magnesium | |
| Fibronectin | Amyloid-β precursor protein | |
| Vitronectin | Factor XIII | |
| Coagulation Factors | $\alpha_1$-protease inhibitor | |
| Factor S | IL-1β | |
| Factor V | Chemokines | |
| Factor XI | RANTES (CCL5) | |
| Mitogenic Factors | MIP-1α (CCL3) | |
| Platelet-derived growth factor | MCP-3 | |
| TGF-β | GRo-α | |
| Endothelial cell growth factor | Platelet factor-4 | |
| Epidermal growth factor | NAP2 (CXCL7) | |
| Insulin-like growth factor-1 | IL-8 | |
| Vascular endothelial growth factor | | |
| Albumin | | |
| Immunoglobulins | | |
| Granule membrane-specific proteins | | |
| P-selectin (CD62P) | | |
| CD63 | | |
| GMP33 | | |
| Fibrinolytic inhibitors | | |
| Plasminogen activator inhibitor-1 | | |
| $\alpha_2$-Plasmin inhibitor | | |

(Ambrosio *et al*., 2012; Gros *et al*., 2015; Koudouovoh-Tripp and Sperner-Unterweger, 2012; Mumford *et al*., 2015; Smyth *et al*., 2009)

Platelet δ-granules are responsible for the storage of 5-HT, the accumulation of which is derived from overflow of its production by enterochromaffin cells of the digestive tract (Jedlitschky *et al*., 2012; Mumford *et al*., 2015). In addition, δ-granules act as storage sites for pyrophosphates, calcium, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) (Ambrosio *et al*., 2012; Gobbi *et al*., 2003; Jedlitschky *et al*., 2012; Mumford *et al*., 2015). The contents of these granules are essential for mediation of sustained platelet activation; the importance of their involvement is evident in patients with bleeding disorders as a result of a deficiency in δ-granules, or their contents (Jonnalagadda *et al*., 2012).

The membrane proteins of δ-granules include CD63, which increases on the outer surface of platelets following their exocytosis, as well as those responsible for turnover of δ-granules content, such as the ADP transporter and vesicular monoamine transporter-2 (VMAT-2).

Platelet lysosomes contain the enzyme β-hexosaminidase, as well as other acid glycohydrolases (Ambrosio *et al*., 2012). The role of their release remains unclear, although it is hypothesised that lysosomal enzymes could be important in thrombus remodelling and host defence (Jedlitschky *et al*., 2012; Jonnalagadda *et al*., 2012; Mumford *et al*., 2015).

Platelet granule release requires regulated synthesis of each granule type, in conjunction with a multi-step secretion pathway that comprises of the following components: (i) agonist interaction with surface receptors on the platelet, (ii) intracellular signalling, (iii) alterations in intracellular levels of calcium, phosphates and kinases, (iv) remodelling of the cytoskeleton and finally (v) fusion of the granule and platelet membranes that results in the release of the granule contents (Mumford *et al*., 2015). A diagrammatic representation of this process can be seen in Figure 2.5.

When granules are exocytosed upon platelet activation, integral membrane proteins are inserted into the plasma membrane, altering its composition. Therefore, activated platelets exhibit extra surface markers, such as P-selectin, CD63 and integrin $\alpha_{IIb}\beta 3$ (Jonnalagadda *et al*., 2012; Smyth *et al*., 2009). The exposure of P-selectin (CD41a) is important for platelet-leukocyte interaction, which will be discussed in the next section. Stimulation of platelets with low concentrations of weak agonists, such as ADP, results in a greater α-granule-δ-granule release ratio. However, stimulation with strong agonists (such as monoamine-releasing agents) appears to reverse this ratio (Jonnalagadda *et al*., 2012).

### 2.2.2 Platelet modulation of the inflammatory response

The human inflammatory response is highly regulated by an intricate network of control factors. Among these regulatory components, cytokines are most prominent. A dynamic balance exists between pro- and anti-inflammatory cytokines, and under pathological conditions, anti-inflammatory cytokines may not provide sufficient control over pro-inflammatory activities, resulting in a systemic pro-inflammatory state (Opal and DePalo, 2000; Poon *et al*., 2015).

It has been shown that platelets contribute to inflammation in a number of conditions including infections, and in chronic inflammatory disease (Duerschmied *et al*., 2014; Gawaz *et al*., 2005; Smyth *et al*., 2009). Since platelets, upon activation and adhesion, secrete cytokines, chemokines, and other mediators of inflammation stored in their α- and δ-granules, they possess the capacity to initiate, modulate, propagate and resolve the inflammatory response (Gros *et al*., 2015).

Platelets possess the ability to regulate most effector functions of neutrophils and macrophages, such as reactive oxygen species (ROS) production, secretion of neutrophil granule content, and phagocytosis (Duerschmied *et al*., 2014). Evidence of direct contact between platelets and neutrophils/monocytes via P-selectin is also important for the oxidative burst required for activation (Gros *et al*., 2015).

Additionally, while some platelet-derived factors target leukocytes such as neutrophils and monocytes directly, others like 5-HT and platelet-activating factor also act on platelets themselves, thus amplifying their regulatory effects on leukocyte recruitment. The relationship between platelets and major leukocytes in the inflammatory response, can be seen in *Figure 2.4*.

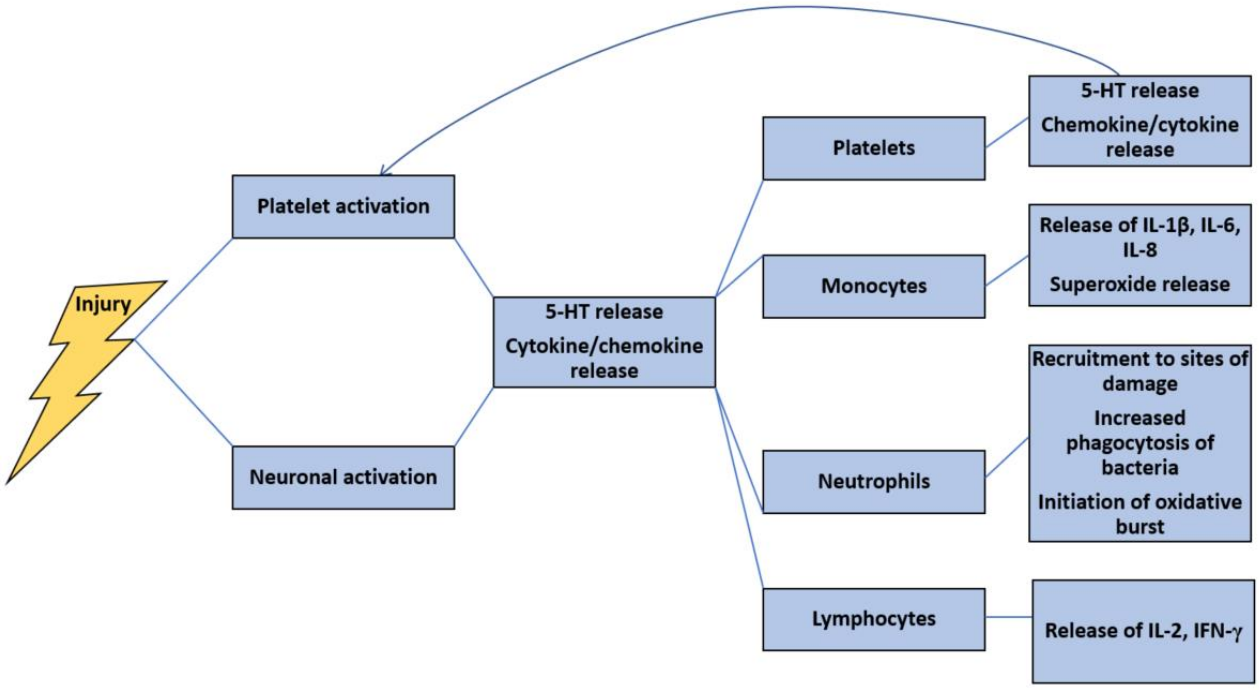



*Figure 2.4: Platelet-platelet and platelet-leukocyte interaction in the inflammatory response (redrawn and modified from Duerschmied et al. (2014) and Gros et al. (2015))*

The immunomodulatory ability of platelets is important in the setting of chronic stress, since we now know that it is characterised by hyperresponsiveness of the stress-immune pathways, which also affect platelet functionality. Since platelet activation involves secretion of 5-HT, chronic stress results in hypersecretion of platelet 5-HT stores. 5-HT is an important activator of the clotting cascade in response to injury; thus, an

excess of 5-HT in circulation stimulates chronic platelet activation via 5-$HT_{2A}$, which subsequently causes blood hypercoagulability and elevated cytokine secretion.

The consequences of chronic platelet activation feed back into the complex cycle of stress-immune crosstalk, contributing to the development of chronic inflammatory disease and anxiety/depression. Since platelets are affected so similarly to serotonergic neurons in this cycle, they have been used as a model for the central serotonergic system in a number of studies, specifically for the study of coagulation in chronic inflammatory illness (Hüfner *et al*., 2014; Silić *et al*., 2012; Soma *et al*., 2016; Van Rooy and Pretorius, 2015) and to better understand 5-HT dynamics (Gobbi *et al*., 2003; Ramström *et al*., 1999), sometimes in response to anxiolytic or anti-depressive treatments (Born *et al*., 1980; Gawaz *et al*., 2005; Jedlitschky *et al*., 2012; Reikvam *et al*., 2012). The next section will highlight the major similarities between platelets and serotonergic neurons and indicate their diagnostic potential in anxiety/depression therapeutics.

### 2.2.3 Structural similarities between platelets and serotonergic neurons

#### *2.2.3.1 Serotonin uptake and storage*

5-HT uptake by platelets is the primary mechanism of 5-HT clearance from the blood. The key difference between platelets and serotonergic neurons, is the inability for platelets to produce 5-HT (Charnay and Léger, 2010; Jacobs and Azmitia, 1992). While 5-HT is synthesised and then stored in vesicles within serotonergic neuron axons and terminal buds, 5-HT storage in platelet δ-granules results from a two-step process: (i) the uptake of 5-HT across the plasma membrane via SERT, and (ii) transport across the δ-granule membrane via VMAT-2. SERT and VMAT-2 are also present in serotonergic neurons and are responsible for reuptake of released 5-HT from the synaptic cleft to limit duration and magnitude of 5-HT signalling, and for 5-HT uptake and storage in neuronal vesicles, respectively. Transport via VMAT-2 is facilitated by an electrochemical proton gradient across the dense body membrane, which is established by vacuolar $H^+$-ATPase (Jedlitschky *et al*., 2012).

Platelets also possess uptake mechanisms for certain amino acid transmitters such as γ-aminobutyric acid, aspartate, glutamate and glycine. Uptake of these factors resembles that of neurons in the CNS (Rainesalo *et al*., 2005) – this further qualifies platelets as a model system for the central serotonergic system.

#### *2.2.3.2 Serotonin release and receptors*

The release of 5-HT by platelets promotes platelet functions via the 5-HT receptor, 5-$HT_{2A}$. When platelets adhere and aggregate at a site of vessel injury, 5-HT is released, which directly accelerates platelet aggregation and vasoconstriction, and potentiates the response of platelets to other agonists such as adenosine diphosphate, collagen, and thromboxane $A_2$ (Hoirisch-Clapauch *et al*., 2014; Jedlitschky *et al*., 2012).

Sustained stimulation of platelet 5-HT receptors by elevated circulating 5-HT results in receptor desensitization and decreased expression. Likewise, excess 5-HT in the CNS acts as negative feedback on

autoreceptors on serotonergic neuron terminals, decreasing both 5-HT release by presynaptic neurons and decreased receptor expression on postsynaptic serotonergic neurons (Charnay and Léger, 2010). These alterations may be responsible for the delayed response to anxiolytic or antidepressant treatment in some patients.

The major similarities between platelets and central serotonergic neurons are summarised in *Figure 2.5*.

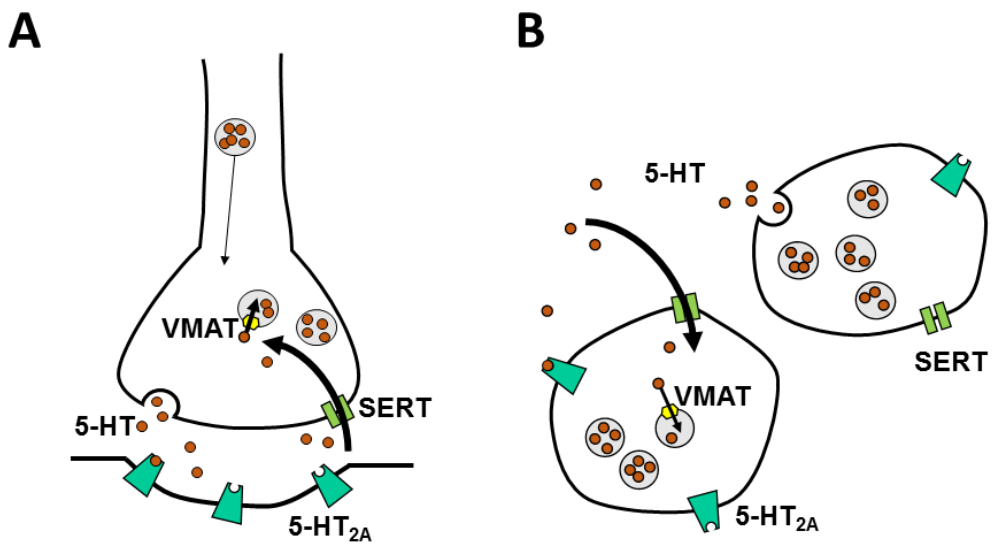



*Figure 2.5: Key structural similarities between (A) central serotonergic neurons and (B) platelets (redrawn and modified from Jedlitschky et al. (2012))*

*2.2.3.3 Platelets in anxiety/depression*

Not only are platelets activated by agonists (ADP, calcium, heparin etc) and physical stress, but also by mental stress (Koudouovoh-Tripp and Sperner-Unterweger, 2012), highlighting another link between platelets and central serotonergic neurons. Catecholamines, the main neurotransmitters in the SAM axis, activate platelets via adrenergic receptors. HPA axis hyperactivity has also been shown to stimulate platelet 5-HT release, as well as increase pro-inflammatory cytokine secretion (Leonard, 2014). Additionally, platelets are activated by certain lifestyle factors that contribute to chronic systemic inflammation, such as smoking, excess alcohol and drug use, and poor dietary habits.

Since chronic stress leading to the development of chronic inflammation and mental health disorders, results in 5-HT hypersecretion and downregulation of SERT and 5-HT receptors (Maes *et al*., 2011, 1998; Silić *et al*., 2012), one would expect to observe similar effects in peripheral blood platelets isolated from anxious or depressed individuals. This supports the idea that platelets could potentially be employed as an easily accessible, effective model with which to diagnose anxiety type (acute vs chronic) or depression status, based on maladaptations in intraplatelet 5-HT levels and receptor expression. These parameters may also prove useful in determining disease progression and potential targets for treatment.

Staying with the scope of this thesis, depression and anxiety disorders are most often treated with pharmaceuticals aimed at increasing either 5-HT levels, or duration of its synaptic availability, or both. More recently, some focus has also been on natural products with demonstrated central bioavailability. In the next few sections, I provide an overview of the most common pharmaceutical treatment types and their mechanisms of action, followed by a short reference to one natural product that we have been studying in this context for many years.

## 2.3 Therapeutic interventions

The inflammation associated with anxiety and depression is attenuated by anxiolytic and antidepressant drugs (Abdel-Salam *et al*., 2004; Sawynok *et al*., 2001). Conversely, clinical evidence has shown that non-steroidal anti-inflammatory drugs such as cyclooxygenase-2 (COX-2) inhibitors and infliximab exert anti-depressive effects, and have even been found to enhance the therapeutic response when administered in combination with antidepressants (Krause *et al*., 2012; Müller, 2010).

The bi-directional effects of anxiolytics/antidepressants are of great interest in the context of chronic inflammatory disease, where a self-propagating cycle exists in terms of inflammation and illness. Two of the major classes of drugs employed in the attempted treatment of anxiety and depressive disorders are SSRIs and MRAs. The beneficial effects of these pharmaceuticals will be discussed in the following sections.

### 2.3.1 Selective serotonin reuptake inhibitors and monoamine-releasing agents

#### *2.3.1.1 Serotonergic system modulation by serotonin reuptake inhibition*

Inhibition of 5-HT uptake was first related to antidepressant actions in 1975 by Wong *et al*. However, the first pharmaceuticals designed to target reuptake also targeted 5-HT receptors, causing adverse effects such as sedation and cardiac arrest (Hiemke and Härtter, 2000). Therefore, the development of safer drugs that exclusively blocked 5-HT uptake was of great priority.

Consequently, SSRIs – which have high affinity for SERT and low affinity for receptor sites – were developed (Duerschmied *et al*., 2014, Hoirisch-Clapauch *et al*., 2014), and are among the most prescribed drugs today (Gobin *et al*., 2014). Although SSRIs have better overall safety and tolerability than older counterparts, their side-effects include sexual dysfunction, sleep disturbance and weight gain (Hiemke and Härtter, 2000; Millan, 2004).

Citalopram has shown to have the highest selectivity for SERT out of all SSRI medications (Hiemke and Härtter, 2000), and has been used to treat conditions ranging from minor to major depression, anxiety, panic disorders, and obsessive-compulsive disorders (Gobin *et al*., 2014). In blocking 5-HT reuptake from synaptic clefts within the CNS, SSRIs exert their effects by facilitating longer 5-HT availability and increased receptor binding (Matthäus *et al*., 2016). This is beneficial in the context of 5-HT depletion due to hypersecretion, where both 5-HT concentration and receptor expression are low.

Importantly, patients on SSRI treatment have been found to be at increased risk for abnormal bleeding, but at decreased risk of arterial occlusive events (Hoirisch-Clapauch *et al*., 2014; Jedlitschky *et al*., 2012; Reikvam *et al*., 2012), due to the fact that SSRIs prevent platelet 5-HT uptake via SERT. This ultimately decreases platelet-platelet communication in the context of activation for coagulation purposes. In human studies, all SSRIs have shown a significant decrease in platelet 5-HT content after several weeks of treatment, reaching levels below ±10% of the pre-treatment levels (Hoirisch-Clapauch *et al*., 2014).

*2.3.1.2 Anti-inflammatory properties of SSRIs*

*In vitro* studies with human whole blood have shown that SSRI antidepressants inhibit the production of pro-inflammatory cytokines IL-1, -2, and -6, and TNF-α, while stimulating the secretion of IL-10 (Berk *et al*., 2013; Gobin *et al*., 2014; Leonard, 2014; Maes *et al*., 1997). These effects are independent of the action of SSRIs on SERT, the mechanisms of which will be discussed next.

A proposed mechanism of action behind the anti-inflammatory effects of SSRIs is the inhibition of interferon-γ secretion, which in turn impedes the influx of calcium ions into immune cells. Calcium is necessary for the activation of the JAK-STAT pathway; thus, reduced activation of this pathway results in decreased release of pro-inflammatory cytokines (Leonard, 2014).

Studies performed on various SSRIs noted the following: (i) long-term administration in rats attenuated LPS-induced depressive behaviour and neuroendocrine alterations; (ii) administration of sertraline is accompanied by attenuation of inflammatory response activation, which was indicated by reduced pro-inflammatory cytokine levels; (iii) chronic treatment with imipramine demonstrated a reduction in immune activation in rats subjected to a chronic, mild stress model of depression (Schiepers *et al*. 2005).

*2.3.1.3 Monoamine-releasing agents*

Despite the improved safety profiles of current SSRIs, several weeks of administration are required prior to the expression of beneficial effects, since therapeutic efficacy relies on alterations in receptor density and serotonergic neurogenesis (Millan, 2004). A reduction in undesired side effects is also of importance, as these compromise compliance and interfere with the therapeutic outcomes of SSRI treatment (Hiemke and Härtter, 2000).

Attenuation of low 5-HT signalling dynamics may also be achieved through increasing 5-HT:SERT and 5-HT:receptor ratios. MRAs, capable of altering this ratio, are considered the third – and most recent – generation of antidepressants. MRAs have been shown to have improved tolerability, increased efficacy, shorter delay in clinical efficacy, and effective anxiolytic and antidepressant effects (Millan, 2004).

MRAs enhance SERT-mediated exchange and release of 5-HT, by shunting available tryptophan into the tryptophan hydroxylase pathway, resulting in greater 5-HT synthesis (Capuron *et al*., 2003). In addition, MRAs prevent uptake of 5-HT into storage vesicles, through inhibition of VMAT-2, and cause release of this

neurotransmitter through reverse-transport at VMAT-2 (Simantov, 2004). Some potent MRAs, such as methamphetamines, are also capable of binding to SERT, which further potentiates the effects of 5-HT as discussed previously (Simantov, 2004).

Overlooking the adverse effects of SSRIs, one can appreciate the therapeutic potential of a drug that possesses both mild MRA and mild SSRI properties, as it may provide immediate beneficial effects through 5-HT release while neuronal plastic remodelling occurs under the influence of SSRI treatment. In addition, the synergistic effects of monoamine release, together with 5-HT reuptake inhibition, may lend itself to greater beneficial outcomes in terms of treatment of mental health disorders as well as chronic stress-induced inflammation. A summary of the targets of these common antidepressants/anxiolytics in the complex cycle of chronic stress and chronic inflammatory disease can be seen in *Figure 2.6*.

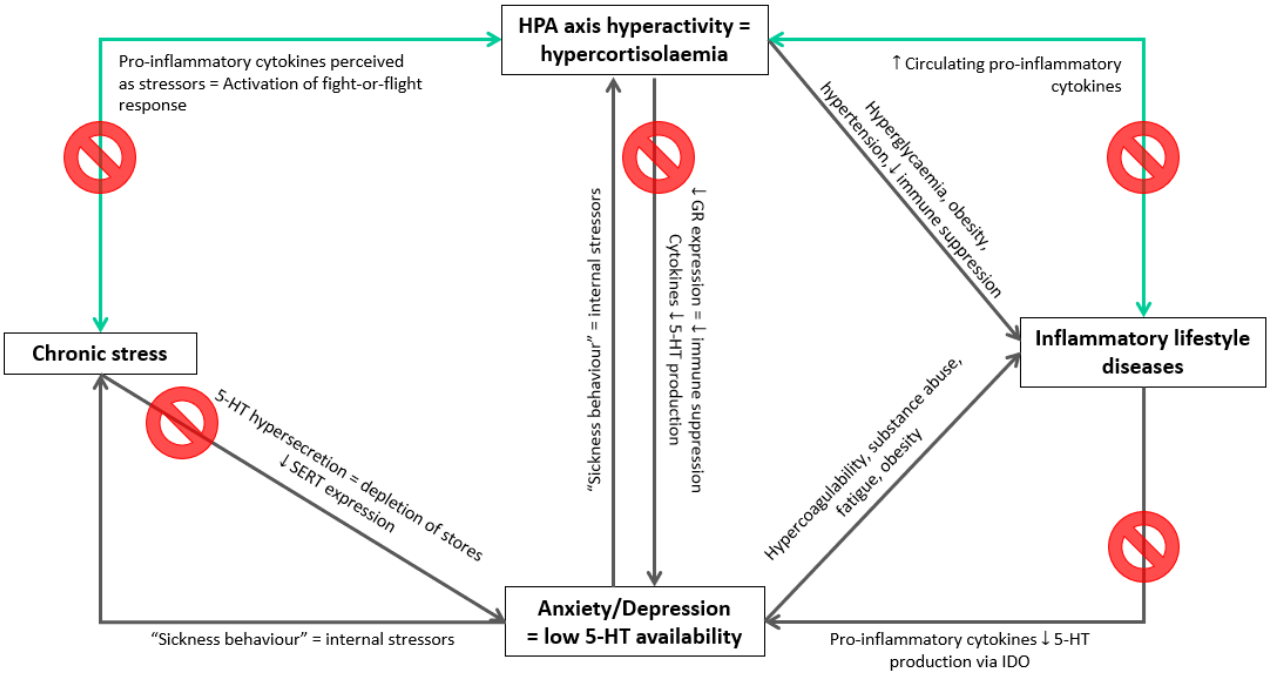



*Figure 2.6: Therapeutic targets of SSRIs and MRAs in the treatment of chronic stress-induced inflammatory disease*

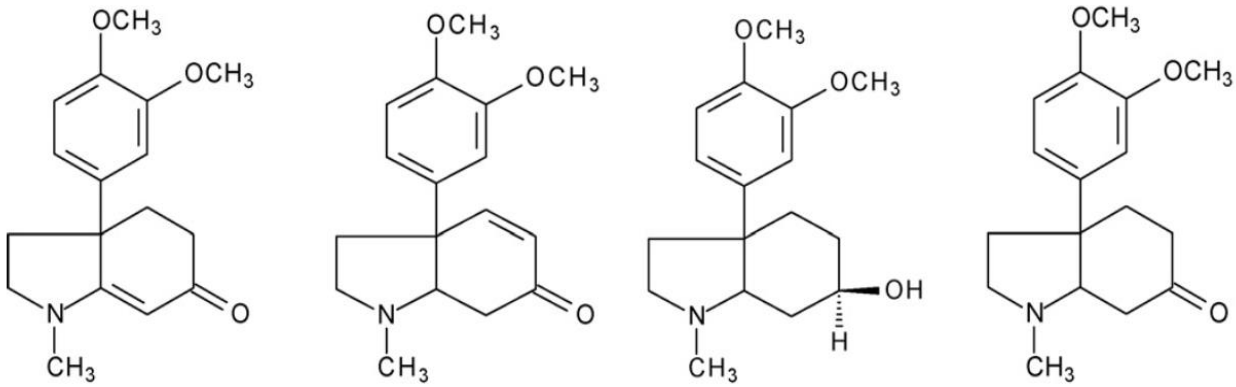
The last section of this review highlights the therapeutic potential of *Sceletium tortuosum* in the context of inflammatory mental health disorders and chronic inflammatory disease.

### 2.3.2 *Sceletium tortuosum*

*Sceletium tortuosum*, known as *kanna* in Nama and *kougoed* in Afrikaans, has one of the oldest histories of use of any South African medicinal plant (Gericke and Viljoen, 2008; Shikanga *et al*., 2012). The earliest written records of *Sceletium tortuosum* use date back to 1662 and 1685 (Murbach *et al*., 2014). The plant is most commonly chewed but can also be consumed as a tea or snuff and smoked. It was originally used by

indigenous people for toothache, and relief from hunger and abdominal pain (Loria *et al*., 2014; Shikanga *et al*., 2012).

In the western setting, *Sceletium tortuosum* first gained popularity due to anecdotal claims of mood-elevating, anxiolytic properties, especially following fermentation of the plant material (Patnala and Kanfer, 2009; Smith *et al*., 1996). Scientific investigation of these claims has shed light on the mechanisms of action behind *Sceletium's* effects on the CNS. This section will highlight the main findings from major studies involving a high-mesembrine (*Figure 2.7*) *Sceletium tortuosum* extract (Trimesemine™) and indicate further research areas to elucidate exact effects and mechanisms of action of this traditional medicine.




Δ7-mesembrenone mesembrenone mesembranol mesembrine

*Figure 2.7: The four major alkaloids that define the alkaloid composition of Sceletium tortuosum (modified from Patnala and Kanfer (2009))*

Mesembrine is one of the most pharmacologically-active alkaloidal components of *Sceletium tortuosum*, and was recently shown to possess cytoprotective and mild anti-inflammatory properties in the setting of acute inflammation in the peripheral compartment, completely inhibiting the approximately 40% loss in monocytic cell viability after exposure to LPS and limiting pro-inflammatory cytokine production in this context, although not inhibiting it entirely (Bennett and Smith, 2018). In addition, Trimesemine™ was found to directly modulate adrenal steroidogenesis, effectively inhibiting glucocorticoid production (Swart and Smith, 2016). These *in vitro* findings indicate that *Sceletium* supplementation may be beneficial in the context of chronic inflammation-related illnesses.

In terms of central action of the plant, we have shown that Trimesemine™ exerts anxiolytic effects in a rat model of psychological stress (Smith, 2011). These effects were confirmed in a rat model of nociception, anxiety, depression and ataxia (Loria *et al*., 2014), where *Sceletium* extract supplementation was found to exert beneficial effects in terms of mood elevation and decreased nociceptive responses, when compared to suitable SSRI and MRA controls.

The effect of the *Sceletium tortuosum* has, to date, been tested in a few human clinical trials (Nell *et al*., 2013; Smith *et al*., 1996; Terburg *et al*., 2013). In a recent placebo-controlled clinical trial, unsolicited positive comments from individuals supplemented with a commercial *Sceletium* extract, Zembrin®, included feeling generally "better", coping well with stressful situations, and improved sleep at night. The study showed that doses of 8mg and 25mg, taken once daily for three consecutive months, were well tolerated by the human body in the context of physical examination and laboratory assessments (haematology, urinalysis and biochemistry) and the recording of adverse events (Nell *et al*., 2013). However, it was stated that further clinical studies should be conducted to investigate the effects of *Sceletium* on cognitive function, anxiety and mood. Subsequently, another paper on the same product reported attenuated amygdala reactivity to fearful faces after a single dose of low-mesembrine *Sceletium* extract (Terburg *et al*., 2013).

A recent *in vitro* study by our group in human astrocytes and mouse hippocampal cells further elucidated the mechanisms of action behind the anxiolytic effects of Trimesemine™, indicating that it acts primarily as an MRA, which suggests that the more frequently reported SSRI function may be a secondary mechanism (Coetzee *et al*., 2016). These results, in addition to the confirmed anti-inflammatory effects of Trimesemine™, indicate that this plant extract is ideal for studying the serotonin-immune link, allowing for more detailed interpretation of generated data.

The promising effects elucidated for a high-mesembrine *Sceletium tortuosum* extract warrant further research into the other major alkaloids present in the plant. Preliminary research by our group using a high Δ7-mesembrenone extract has shown potential anti-cancer effects (unpublished data). Since the relationship between inflammation and cancer is well known (Colotta *et al*., 2009; Dhabhar, 2009; Mantovani *et al*., 2008; Reiche *et al*., 2004), this further supports the anti-inflammatory potential of *Sceletium tortuosum*.

*Sceletium tortuosum's* apparent immunomodulatory properties may be attributed to many mechanisms. Firstly, changes in immune activity and cytokine production after treatment may be secondary to changes in neurotransmitter activity, as brain-immune communication occurs through monoaminergic signalling (Basu and Dasgupta, 2000; Gobin *et al*., 2014). Secondly, *Sceletium* may attenuate cytokine-induced alterations in GC receptor expression, thereby ceasing cytokine-induced GC resistance in both the hypothalamus and pituitary gland (Carvalho and Pariante, 2008; Kiecolt-Glaser *et al*., 2015). In doing so, *Sceletium* may restore GC-mediated negative feedback inhibition of the HPA axis. A final mechanism by which *Sceletium* may reverse the central effects of cytokines may be through inhibition of cytokine-induced indoleamine-2,3-dioxygenase activation (Arreola *et al*., 2015; Capuron *et al*., 2003; Dahl *et al*., 2014). As mentioned previously, overstimulation of indoleamine-2,3-dioxygenase by cytokines has been implicated in HPA-axis and 5-HT disorders associated with mental health disorders.

From this data, I conclude that *Sceletium* extract in moderation is safe for human consumption. Furthermore, in light of all the much-desired effects it may affect in individuals afflicted with anxiety and/or depression, the therapeutic potential of this plant should be further developed.

## 2.4 Hypothesis and Aims

I hypothesised that a high-mesembrine and high-Δ7 mesembrenone *Sceletium tortuosum* extract will beneficially modify both the inflammatory immune system and the serotonergic system, in the context of chronic stress-related disorders. Secondly, I hypothesised that peripheral blood platelets are a suitable model of the central serotonergic system in this context.

To test this hypothesis, the following aims were formulated:

- Evaluation of the immunomodulatory properties of *Sceletium tortuosum* in the central nervous system
- Assessment of the suitability of the platelet model to mimic the serotonergic system
- Elucidation in more detail, the manner in which *Sceletium tortuosum* relieves anxiety via modulation of neuro-immune crosstalk

# Chapter 3 – Experiment I

In this chapter, I present data in manuscript format, as submitted for publication in *Journal of Physiology and Biochemistry* (Impact Factor 2.44). I was invited to write this manuscript for a special issue, following presentation of these data at an international conference in 2017 (Conferences on Trans-Pyrenean Investigations in Obesity and Diabetes: 14th French-Spanish Meeting). Although the journal invitation specified obesity as context for data presentation, these data is also relevant to other inflammatory conditions, such as anxiety.

At this point, I would like to acknowledge two international collaborators – Prof Victor López and his MSc student Arno van Camp (University San Jorge, Zaragoza, Spain) – for their contribution to this paper. I completed the astrocyte experiments, while they contributed their data on neural enzyme inhibition by the extracts used.

# *Sceletium tortuosum* may delay chronic disease progression via alkaloid dependant antioxidant or anti-inflammatory action

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## Abstract

The link between obesity-induced systemic inflammation and decreased insulin signalling is well-known. It is also known that peripherally-produced inflammatory cytokines can cross the blood-brain barrier, resulting in the release of neurotoxins that can ultimately lead to demise of central nervous system integrity. A high-mesembrine *Sceletium tortuosum* extract was recently shown to possess cytoprotective and mild anti-inflammatory properties in monocytes and to target specific p450 enzymes to reduce adrenal glucocorticoid synthesis. This is significant since the aetiology of both obesity and diabetes is linked to inflammation and excess glucocorticoid production. Given the interlinked nature of glucocorticoid action and inflammation, central immunomodulatory effects of two *Sceletium tortuosum* extracts prepared by different extraction methods were investigated. Human astrocytes were pre-treated for 30 minutes, before exposure to *Escherichia coli* lipopolysaccharide for 23.5 hours (in the presence of treatment). Cytotoxicity, mitotoxicity and cytokine responses (basally and in response to inflammatory stimulus) were assessed. In addition, total polyphenol content, antioxidant capacity and selected neural enzyme inhibition capacity were assessed for both extracts. The high-mesembrine *Sceletium* extract exerted cytoprotective and anti-inflammatory effects. In contrast, the high Δ7-mesembrenone extract, rich in polyphenols, exhibited potent antioxidant effect, although with relatively higher risk of adverse effects with overdose. We conclude that both *Sceletium tortuosum* extracts may be employed as either a preventative supplement or complimentary treatment in the context of obesity and diabetes; however, current data also highlights the impact that extraction methods can have on plant product mechanism of action.

## 3.1 Background

The prevalence of diabetes worldwide is predicted to reach 366 million by 2030 (Wild *et al*., 2004). The major focus of diabetes management is glycaemic control, which commences relatively late in the developmental time frame of the disease. In contrast, oxidative stress and chronic systemic inflammation are present much earlier and responsible for the development of widespread insulin resistance, ultimately resulting in the establishment of type 2 diabetes (T2D) (Shoelson *et al*., 2006).

In addition, peripheral inflammation is linked to cognitive decline, via crosstalk with neuroinflammatory processes. This has been confirmed by multiple studies, which have reported an increased risk of dementia associated with obesity (Anstey *et al*., 2011; Hassing *et al*., 2010; Nepal *et al*., 2014). This phenomenon has been termed "type III diabetes" (T3D) (De La Monte, 2008) and results from overlapping pathways of inflammation, oxidative stress and mitochondrial dysfunction, which form the basis of obesity, type II diabetes (T2D), and neurodegeneration (Pugazhenthi *et al*., 2017; Tucsek *et al*., 2014).

T3D results from peripheral inflammation, which triggers neuroinflammatory responses via gene expression profiling (Thomson *et al*., 2014). In addition, inflammation within the CNS is attributed to damage to the blood-brain barrier (BBB), resulting from migration of leukocytes into the central compartment. Compromised BBB integrity has been observed in conjunction with hippocampal-dependent cognitive decline in a rat model of diet-induced obesity (Tucsek *et al*., 2014).

Both inflammation and oxidative stress seldom occur in isolation and are characteristic of underlying pathology. The relationship between the two is often circular, with no determinable starting point: inflammation may cause oxidative stress in some settings, while in other cases, the converse occurs (Belanger and Magistretti, 2009). Relevant to the topic of diabetes, antioxidant treatment, have been shown to attenuate the harmful effects of high glucose exposure *in vitro* in a simulated blood brain-barrier model (Allen and Bayraktutan, 2009).

The effectiveness of plant medicines with anti-inflammatory capacity in the context of chronic inflammatory disease has also been the focus of many research groups for some time (Ku *et al*., 2014; Petersen and Smith, 2016; Williams *et al*., 2013). One of these potential plant medicines, *Sceletium tortuosum,* was traditionally used by the Khoisan people of Southern Africa for pain relief (Harvey *et al*., 2011; Patnala and Kanfer, 2013), but its commercial availability has significantly increased recently after reports suggest that it may have anti-depressive and anxiolytic properties (Coetzee *et al*., 2016; Smith, 2011).

A high-mesembrine *Sceletium tortuosum* extract was recently shown to possess cytoprotective and mild anti-inflammatory properties in the setting of acute inflammation in the peripheral compartment (Bennett and Smith, 2018). In addition, it has also been shown to target specific enzymes in the adrenal cortical steroid synthesis pathway, to reduce glucocorticoid synthesis (Swart and Smith, 2016). In the context of diabetes

and obesity, this is significant since the aetiology of both conditions is linked to chronically-elevated pro-inflammatory cytokine and glucocorticoid levels (Hotamisligil, 2006; Pugazhenthi *et al*., 2017; Shoelson *et al*., 2006).

Given the illustrated benefits which relate to chronic inflammatory diseases such as diabetes, and given *Sceletium tortuosum's* known psychoactive nature, we hypothesised that the plant could potentially alleviate neuroinflammation and central oxidative stress associated with chronic inflammatory illnesses.

The aims of the current study were therefore, to determine the effects of two different *Sceletium tortuosum* extracts (varying in alkaloidal composition) on human astrocyte viability, both basally and in the presence of an acute pro-inflammatory stimulus (*Escherichia coli* LPS). Furthermore, differences in the functional capacity of these neuroimmune cells before and after pre-treatment with *Sceletium tortuosum* extracts were assessed in terms of their capacity for inflammatory cytokine secretion. To further elucidate the mechanisms of action by which different *Sceletium* extracts exert beneficial effects, their inhibition of selected neural enzymes was assessed. Antioxidant properties of extracts were assessed in terms of total polyphenol content and antioxidant capacity, as well as their effect on astrocyte mitochondrial reductive capacity.

## 3.2 Methods and Materials

### 3.2.1 Extract preparation and characterisation

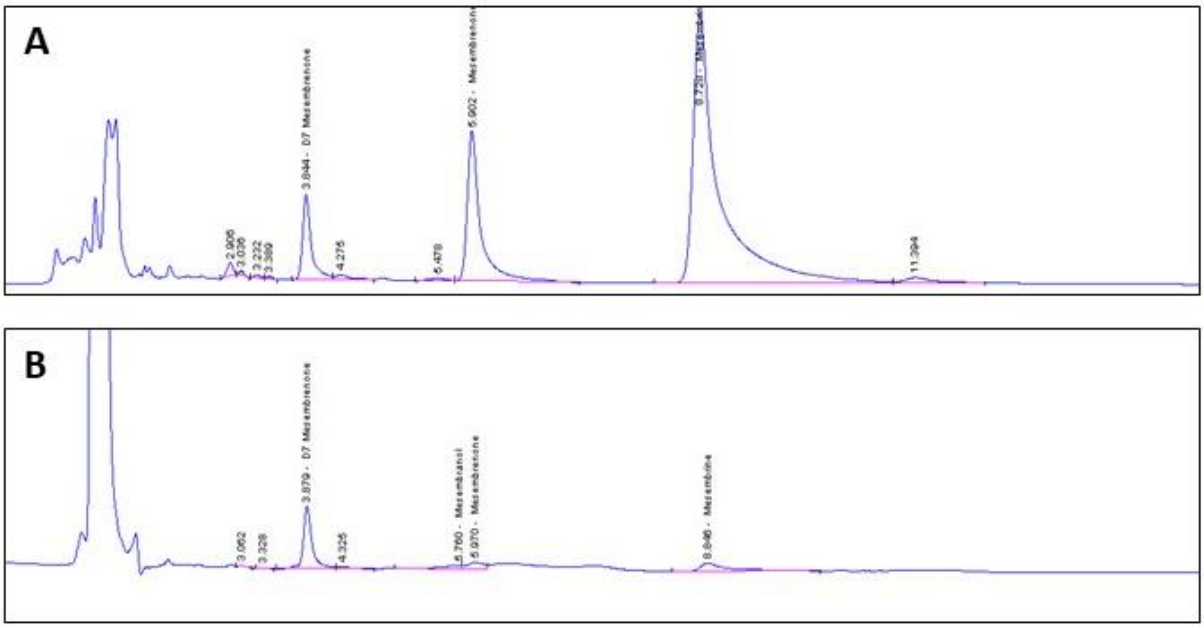


*Figure 3.1:* High-performance liquid chromatography *(HPLC) characterisation of the major alkaloids present in (A) high-mesembrine extract (extract A) and (B) high-Δ7-mesembrenone extract (extract B)*

The relative composition of each extract can be seen in Figure 3.1, with quantification of relative and total alkaloid content presented for comparison in Table 3.1. Extract A is an extract prepared from *Sceletium tortuosum* that was selectively propagated to achieve high mesembrine content, while extract B was more

intensively extracted using different solvents, to achieve particularly high levels of Δ7-mesembrenone, which is usually contained in very low concentrations in *Sceletium* plants. Both extracts (batch numbers for extracts A and B: DV SCITRI:E 591/016 and DV SCDL7:E 525/016 respectively) were prepared by Verve Dynamics™ (Somerset West, South Africa) using proprietary methods and kindly donated by Mr Richard Davies for the purposes of this study.

Table 3.1: Alkaloidal composition of *Sceletium tortuosum* extracts

| Extract | Total alkaloids (mg AE/g) | Total alkaloids (% AE) | Total alkaloids (ppm) | Mesembrine (%) | Mesembrenone (%) | Δ7-Mesembrenone (%) |
|---|---|---|---|---|---|---|
| **A** | 59.81 | 5.981 | 59812 | 68.4 | 19.8 | 7.3 |
| **B** | 16.32 | 1.632 | 16317 | 22.4 | 11.5 | 53.2 |

AE = Atropine Equivalents

### 3.2.2 Cell culture

#### *3.2.2.1 Preparation of treatment media*

A stock solution of each *Sceletium* extract was made (1mg/ml extract A and 3.7mg/ml extract B), by combining extract powder and pre-warmed serum-free media. The mixtures were vortexed for two minutes and subsequently sterile-filtered using a 0.22µm syringe filter. From the filtered stock solutions, dilution series were performed.

The total alkaloidal content of extract B was matched to that of A. The high dose of extract A was used to represent a supra-physiological dose, while the lower dose has previously shown to be most beneficial in similar *in vivo* models (Coetzee *et al*., 2016).

A 2mg/ml LPS (L4391, Sigma Aldrich) stock solution was prepared in Hank's Buffered Salt Solution (HBSS) and added to wells at a final concentration of 20µg/ml for astrocyte inflammatory stimulation.

#### *3.2.2.2 Cell propagation*

Astrocytes (N7805-100, Life Technologies) of low passage (<5) were thawed and cultured in complete Dulbecco's Modified Eagle Medium (DMEM), containing 10% foetal bovine serum (FBS) and 1% Penicillin-Streptomycin, and subsequently seeded into a 48-well culture plate at a density of 1 x $10^4$ cells/well in serum-free DMEM, and incubated (37°C, 5% $CO_2$) for 24 hours to fully adhere to the plate.

### 3.2.3 *Sceletium* extract treatment intervention

The supernatant was aspirated from each well, and the cell monolayer washed once with Dulbecco's phosphate-buffered saline (DPBS) to remove remaining media residue. A 30-minute pre-treatment period

was then initiated, which involved the addition of the different dosages of each *Sceletium* extract to the respective wells. For this period, serum-free media was added to the control and LPS-control groups.

After the pre-incubation period, LPS was added to the LPS-control wells and LPS-*Sceletium* combination groups, to achieve a final LPS concentration of 20µg/ml. The LPS vehicle was added to all control wells. The cells were incubated for a further 23.5 hours. All experiments were performed at least three times, in duplicate.

### 3.2.4 Propidium iodide viability assay

A 1mg/ml propidium iodide (PI) (P1304MP, Thermofisher Scientific) stock solution was prepared according to manufacturer's instructions. For a 3µM working solution, the stock solution was diluted 1:500 in phosphate buffered saline (PBS).

Following the 24-hour treatment intervention, supernatant was aspirated from each well. The cell monolayer was washed with DPBS before the cells were trypsinized, neutralised and centrifuged at 1500rpm for five minutes at room temperature. The resulting supernatant was discarded, and each astrocyte pellet was resuspended 1ml PI working solution. The samples were incubated at room temperature for 10 minutes in the dark before flow cytometric analysis on BD FACSAria IIu (with *BD FACSDiva v8.1 Software*). Live stained, dead stained, and live unstained controls were included (See Appendix G for a detailed protocol of instrument set up, antibody preparation and sample analysis).

### 3.2.5 XTT viability assay

The 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium- 5-carboxanilide (XTT) assay is a commonly used test method to indirectly measure cell viability, through assessment of mitochondrial reductive capacity. Following the 23.5-hour incubation period, supernatant was removed from each well, centrifuged, aliquoted, and stored at −80°C for subsequent batch analysis.

The astrocyte monolayer was washed twice with DPBS to remove residual *Sceletium* isolate treatment, following which XTT (X4626, Sigma Aldrich) solution (1mg/ml) containing 0.5% phenazine methosulphate (P9625, Sigma Aldrich) was added to each well, and a 4-hour incubation period was initiated in a shaking incubator at 37°C. Following incubation, optical densities were determined at 490-nm using a Universal Microplate Reader (Bio-Tek Instruments, Inc. EL800) and analysed using *KCjunior for Windows Data Reduction Software (v1.41.3).*

### 3.2.6 Cytokine measurement

Cell-free culture supernatant was analysed using a commercial magnetic bead panel assay (custom-designed Milliplex MAP Human Soluble Cytokine Receptor Panel, Merck Millipore) for concentrations of IL-6 and monocyte chemoattractant protein-1 (MCP-1) (Appendix B). The fluorescent signals were analysed with a

Bio-Plex 200 instrument, in conjunction with *Bio-Plex Manager 6.1* software. Cytokine responses were expressed as absolute concentrations in cell culture supernatant.

Quantification of cytokine concentrations was performed based on a standard curve, derived from linear dilution of the manufacturer-supplied cytokine standards. The detection limit was 0.9pg/ml for IL-6 and 1.6pg/ml for MCP-1.

### 3.2.7 Neural Enzyme Inhibition Assay

Acetylcholinesterase (AChE) inhibition was measured using a 96-microplate reader based on Ellman's method (Ellman *et al*., 1961). Tyrosinase inhibition was determined using methods adapted from work by Masuda *et al*. (2005). Appropriate controls were used for all analyses. Data were analysed using *GraphPad v6* to obtain IC50 values.

### 3.2.8 Assessment of anti-oxidant capacity and total phenolic content

Antioxidant capacity of both extracts were determined using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) inhibition assay, which assesses radical scavenging capacity against DPPH radicals as previously described (Cásedas *et al*., 2017, 2016), with ascorbic acid as reference standard. Total polyphenol content was determined by the Folin-Ciocalteu assay, using gallic acid as standard. Total polyphenol content was expressed as mg GAE (gallic acid equivalents) per mg of dried extract.

### 3.2.9 Statistical Analysis

Results are presented as averages and standard error of the means (SEMs). Normality of data distribution was assessed, followed by non-parametric 2-way ANOVA and LSD *post hoc* tests. In cases where Levene's test for homogeneity of variances rejected the null hypothesis, the Games-Howell test was used as a *post hoc test* (*Statistica v.13.2*). Differences were considered significant at $p<0.05$.

## 3.3 Results

### 3.3.1 Neuroprotective effects

The PI exclusion assay illustrated a significant degree of cell death (fewer cells able to exclude the PI stain) in astrocytes after exposure to LPS (Figure 3.2). While extract A afforded complete protection in a dose-dependent manner (Figure 3.2A), extract B was not effective at any dose assessed (Figure 3.2B). It is also of interest to note than while extract A did not affect cell viability in the absence of LPS, the highest dose of extract B appeared to be cytotoxic.

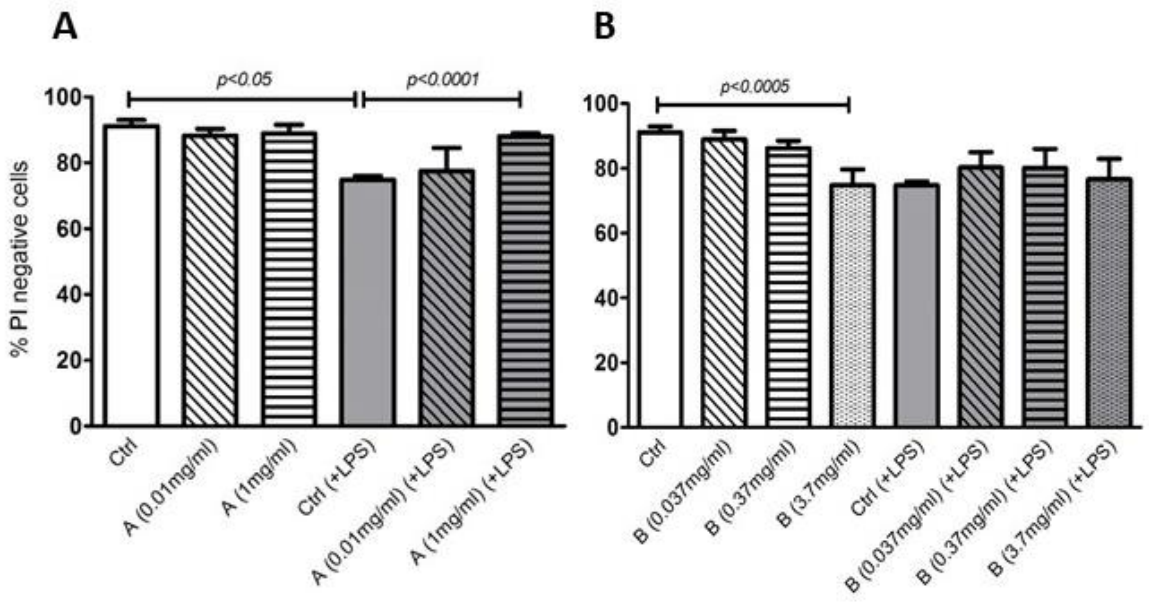


*Figure 3.2: Cell viability determined by propidium iodide assay, following treatment with (A) extract A or (B) extract B with or without LPS stimulation*

### 3.3.2 Anti-inflammatory outcome

The response of astrocytes to the extracts, both in presence and absence of an inflammatory stimulus, was assessed in terms of two major pro-inflammatory cytokines associated with both neuroinflammation and systemic inflammation – IL-6 and MCP-1.

As expected, LPS exposure elicited significant cytokine responses for both IL-6 and MCP-1 (ANOVA main effect, $p<0.005$ and $p<0.0005$ respectively). This response was significantly inhibited by extract A, again in a dose-dependent manner, with the highest dose tested returning cytokine secretion to basal levels (Figure 3.3A and B). In contrast, only the lower doses assessed for extract B inhibited the IL-6 response significantly, with increasing doses suggesting a relatively more pro-inflammatory outcome (Figure 3.3C). Similarly, the MCP-1 response indicated no benefit of extract B at any dose, while higher doses, in fact, elicited an inflammatory response similar in magnitude to the one seen after exposure to LPS (Figure 3.3D).

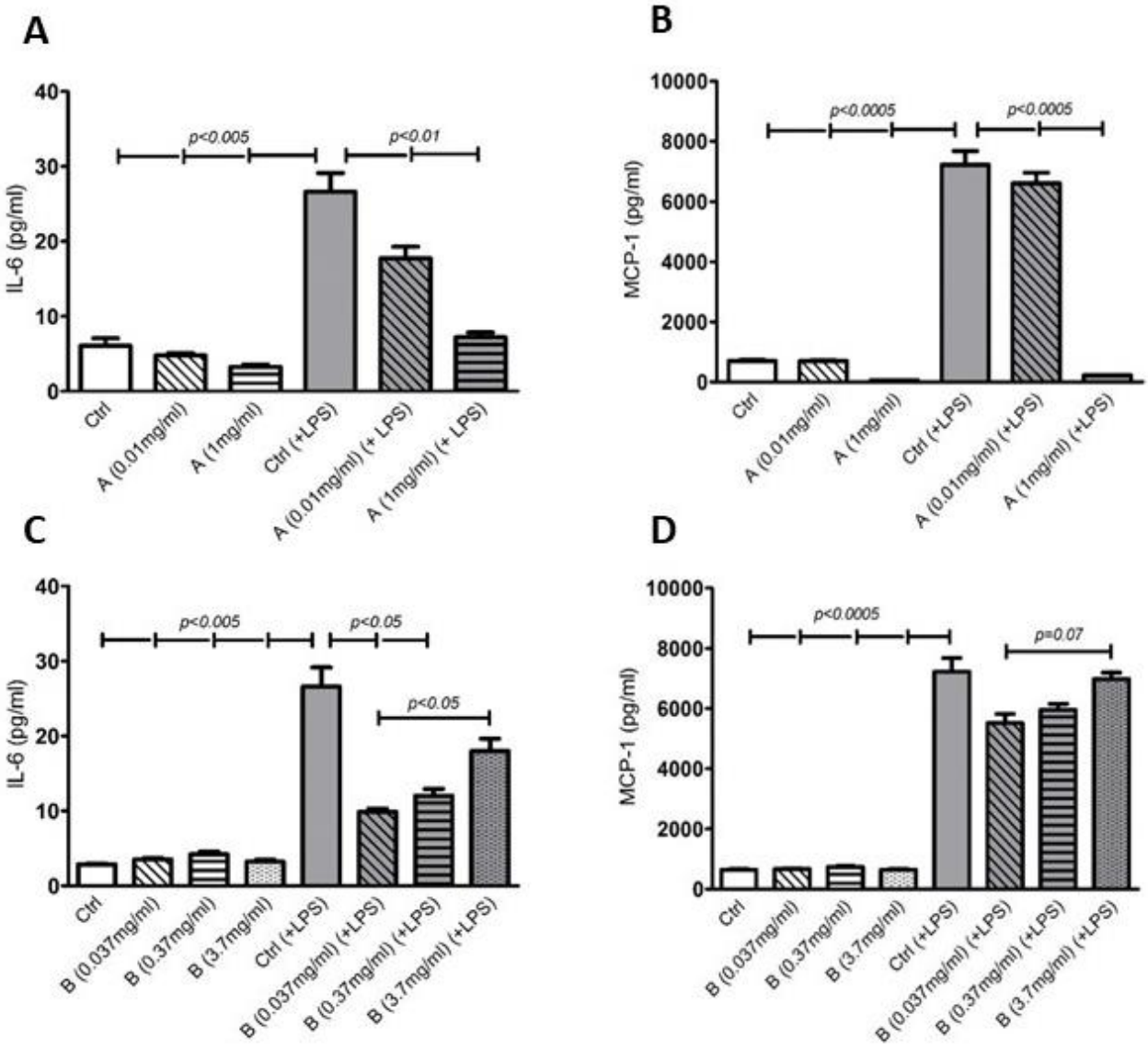


*Figure 3.3: Effect of extract A (A and B) and B (C and D), with/without LPS stimulation, on pro-inflammatory cytokine production by human astrocytes*

### 3.3.3 Neural enzyme inhibition

The effects of the *Sceletium* extracts on two enzymes associated with neurodegeneration, namely tyrosinase and acetylcholinesterase AChE, were assessed. Both extracts exhibited relatively mild inhibitory effects on these neural enzymes when compared to suitable controls (Figure 3.4). Interestingly, while extract A exhibited a higher potency for inhibition of AChE than extract B ($IC_{50}$ 0.299 ± 0.34 vs $IC_{50}$ 0.983 ± 0.16 respectively), the opposite was observed for tyrosinase inhibition ($IC_{50}$ -1.621 ± 0.75 vs $IC_{50}$ -0.5908 ± 0.01 respectively). These results, which suggest as least some degree of neuroprotection – albeit perhaps via different pathways for the different extracts – was confirmed by *ex vivo* exposure of human astrocytes to an inflammatory stimulus.

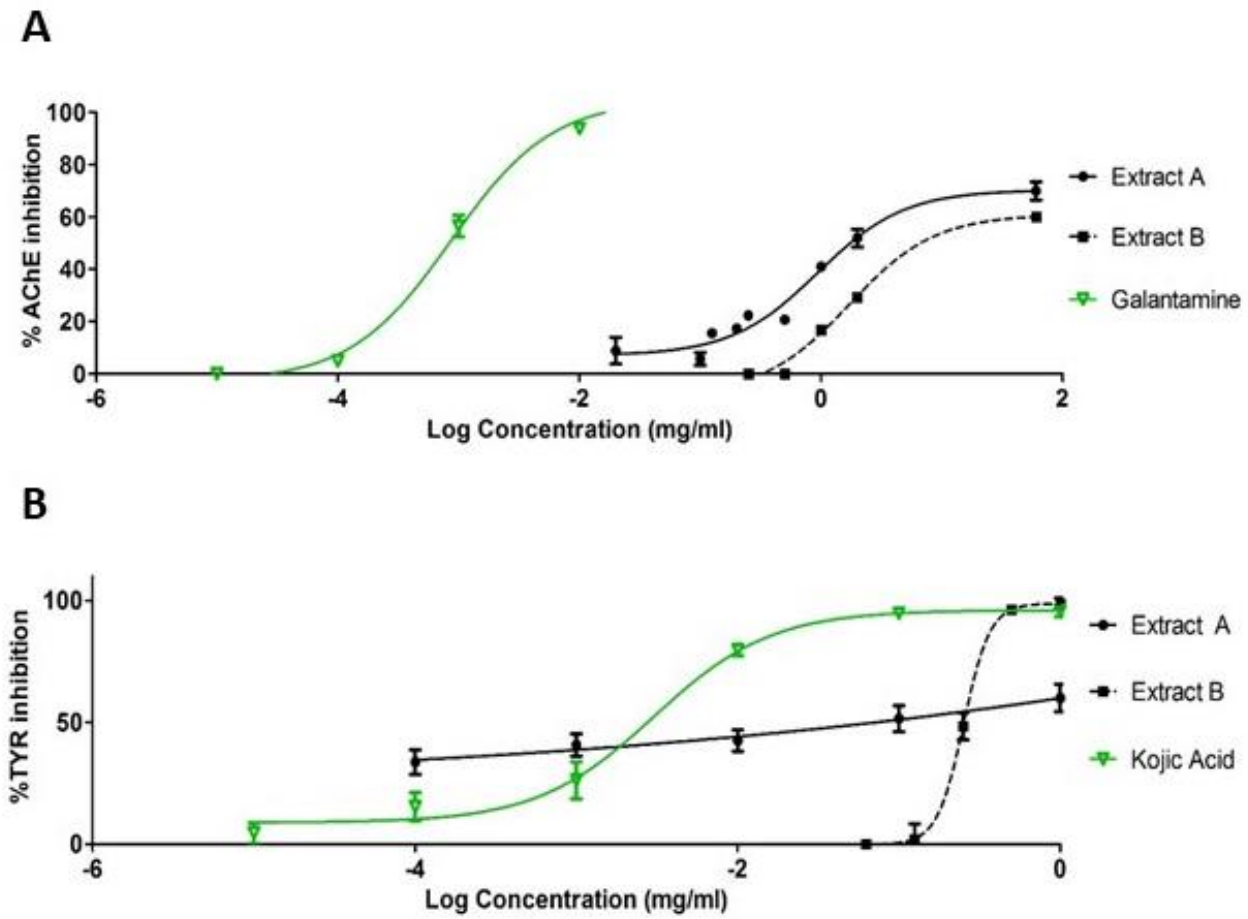


*Figure 3.4: (A) Acetylcholinesterase and (B) tyrosinase inhibition by Sceletium extracts compared to suitable standards*

### 3.3.4 Anti-oxidant outcome

To investigate whether differences in antioxidant capacity may explain the different effects reported for extract A vs. B, antioxidant measures were also assessed. Indeed, total phenolic content of extract B tested much higher than extract A (Figure 3.5A). Similarly, while extract B compared favourably to the ascorbic acid control in terms of DPPH inhibition, extract A did not appear to have significant antioxidant activity in this context (Figure 3.5B).

To put this result into a more physiologically relevant context, the LPS assay was again employed, but this time, mitochondrial functional capacity was assessed. While extract A did not affect this measure in the absence of LPS, extract B significantly compromised mitochondrial reductive capacity at the highest dose (Figure 3.5C and D). In fact, this detrimental effect was similar in magnitude to the reduction in cell functional capacity after exposure to LPS. Neither extract could maintain mitochondrial function in the presence of LPS.

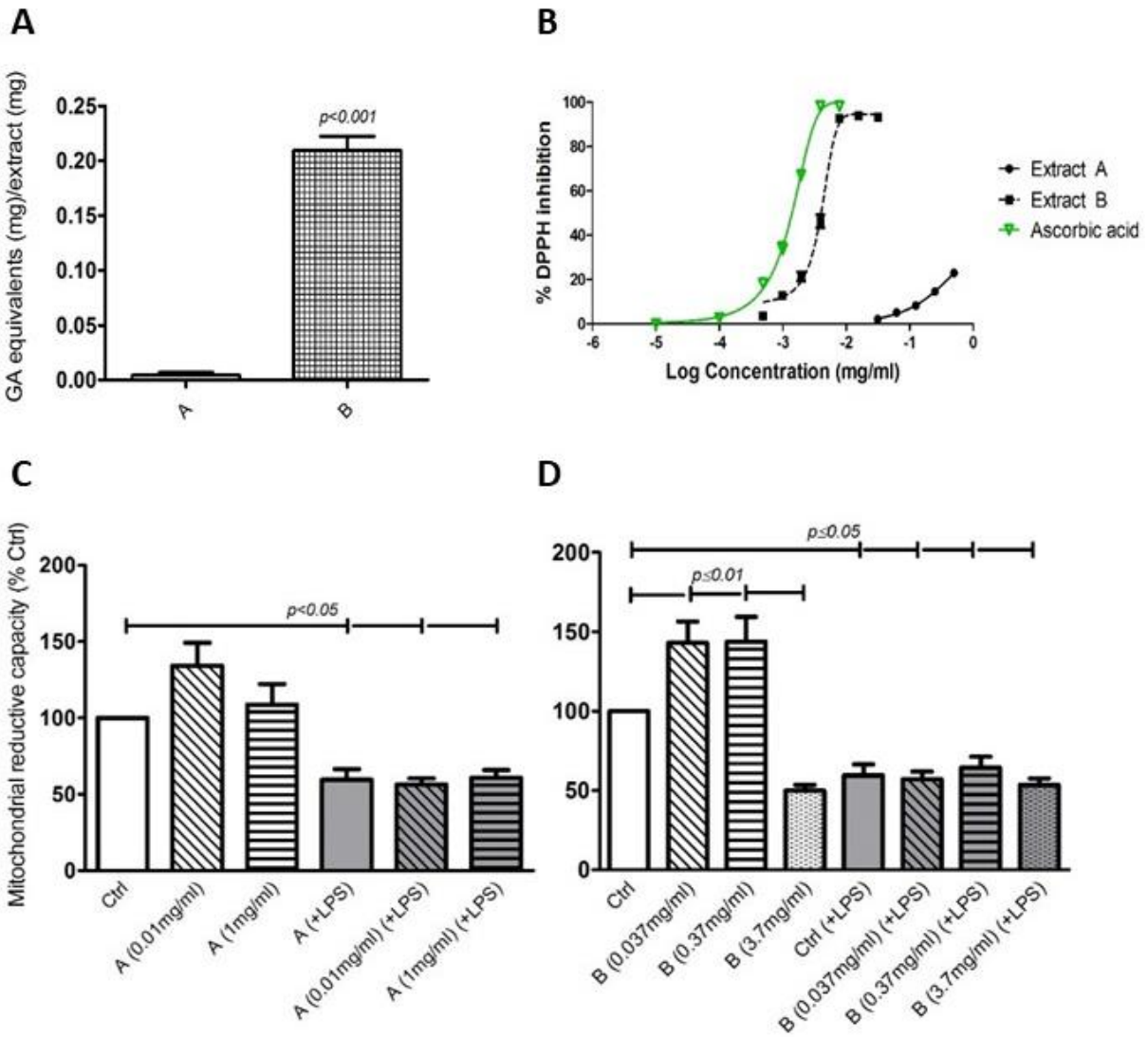


*Figure 3.5: Assessment of antioxidant capacity of extract A and B. (A) total polyphenol content of extracts A and B, expressed as gallic acid (GA) equivalents; (B) antioxidant capacity of extracts A and B, compared to ascorbic acid; (C) astrocyte mitochondrial reductive capacity following treatment with extract A; (D) astrocyte mitochondrial reductive capacity following treatment with extract B*

## 3.4 Discussion

The roles of glial cells in CNS homeostasis maintenance and in the regulation of central innate immune responses are well documented (Streit *et al.*, 2004; Szelényi, 2001; Wrona, 2006). Astrocytes, which

contribute 20-40% of all glial cells, respond vigorously to brain injury, releasing products that can facilitate neuronal protection (Kaushal *et al*., 2015). However, an excessive production of cytokines, chemokines and free radicals may result in functional impairment and neuronal decline (Kaushal *et al*., 2015).

In terms of the validity of the model employed, it was observed that under control conditions (i.e. at 100% viability), astrocytes expressed low levels of IL-6 and MCP-1. This is attributed to a normal physiological response to bi-products of functional metabolic processes in the body, as these cytokines are secreted as mediators of homeostasis maintenance (Balkwill and Burke, 1989). With exposure to LPS, a significant upregulation of cytokine secretion was observed (five-fold and eight-fold for IL-6 and MCP-1 respectively), validating sensitivity of the model to reflect an induced, acute inflammatory response.

When considering effects elicited by the two *Sceletium tortuosum* extracts assessed, clear differences were evident. While astrocyte viability was not affected under basal conditions by either dose of extract A, the highest dose of the Δ7-mesembrenone-rich extract B decreased cell viability by 20%. The decrease in viability was similar in magnitude to the effect of bacterial LPS exposure. While the latter is an expected response due to significant cellular stress caused by a major inflammatory stimulus (Blanco *et al*., 2005), the effect of extract B indicated undesired cell death. Potential reasons for this result were investigated in the context of anti-inflammatory and antioxidant mechanisms.

Firstly, in the context of inflammation, it is important to note that extract A clearly limited endotoxin-induced cytokine production to levels that were not significantly different from control conditions. This indicates a potential mechanism behind the previously-observed cytoprotective effects of extract A (Bennett and Smith, 2018). However, the same effects were not seen in response to extract B treatment, which showed that while it possessed mild anti-inflammatory properties in lower concentrations (0.037mg/ml and 0.37mg/ml), these were not able to limit the inflammatory response to the same extent as extract A. In addition, these effects were not conserved for the highest dose of extract B.

Assessment of neural enzyme inhibition by the extracts shed more light on the differences between the high mesembrine versus the high Δ7-mesembrenone extracts. In terms of AChE inhibition, although relatively mild when compared to galantamine, extract A had higher potency than extract B. A major modulator of peripheral inflammation is the cholinergic pathway, which involves suppression of innate immune responses by acetylcholine. This mechanism inhibits cytokine release by peripheral leukocytes (Gnatek *et al*., 2012). In the CNS, inhibitors of AChE have been shown to enhance the cholinergic anti-inflammatory pathway.

Previous *in vitro* studies have shown that acetylcholine pre-treatment inhibited LPS-induced cytokine release by microglia (Shytle *et al*., 2004). Accordingly, AChE inhibitors have shown to limit astrocyte activation and cytokine production (Gnatek *et al*., 2012). This further supports our interpretation that extract A, which is high in mesembrine, acts as a mild anti-inflammatory agent (Bennett and Smith, 2018).

It was observed that while extract A exerted only mild inhibitory effects in tyrosinase activity, extract B acts as a potent inhibitor of this enzyme, matching the effects of kojic acid at higher doses. This effect suggests that extract B is more antioxidant in nature, in comparison to extract A. Tyrosinase is a copper-containing polyphenol oxidase (i.e. forms part of the reactive oxygen species group), which plays a vital role in melanin pigment formation (Kim and Uyama, 2005). Previously, tyrosinase was associated with Parkinson's disease (Chen *et al*., 2013). Therefore, inhibition of this enzyme may be beneficial in the prevention of neurodegeneration.

The anti-oxidant nature of extract B is further confirmed by assessing its total phenolic content, which was 20-fold higher than that of extract A. In addition, the DPPH inhibition assay indicated that extract B had a similar antioxidant capacity to that of ascorbic acid, a well-documented antioxidant agent (Allen and Bayraktutan, 2009). However, it has been reported that antioxidants in high doses may lose their beneficial, radical-scavenging properties, and may act as pro-oxidants, causing further cellular damage (Burkitt, 2001).

Taken together, this suggests that the 20% reduction in cell viability in response to high-dose extract B exposure can probably be attributed to antioxidant overload-associated cellular damage. This interpretation is in line with the dose-dependent upregulated inflammatory response of the astrocytes exposed to extract B which may have been the result of reactive gliosis in response to oxidative damage.

It was important to assess mitochondrial reductive capacity as an indication of overall cell functionality in the setting of *Sceletium tortuosum* treatment. Mitochondrial dysfunction represents an important link between metabolic syndrome and neurodegeneration, and oxidative stress has been well reported in patients with neurodegeneration, obesity, and T2D, as well as in rodent models of these conditions (Carvalho *et al*., 2012). Mitotoxicity is an important trigger for inflammation (Sorbara and Girardin, 2011), resulting in the secretion of cytokines.

Our results indicate that while extract A had no effect on mitochondrial reductive capacity in either the presence or absence of inflammatory stimulus, treatment with extract B improved this capacity in basal conditions. In line with the indicated anti-oxidant properties of extract B, it was noted that a significant loss of mitochondrial function occurred with exposure to the highest dose of this extract, again indicative of antioxidant overload. Bacterial LPS exposure caused significant mitotoxicity, which was not corrected by the addition of either *Sceletium* extract, indicating that treatment with this natural product may be more beneficial in settings of low-grade inflammation, such as chronic disease, rather than in an acute bout of high-grade inflammation.

It is becoming increasingly important that a standardised composition of the relevant compounds in phytopharmaceutical supplements must be established, from a consumer point of view, as well as for regulatory purposes. However, due to the diversity of compounds present in most plants, coupled with

varying extraction methods, quality control and standardisation of the pharmacologically-active constituents of plant-based supplements is generally not achieved (Patnala and Kanfer, 2013; Shikanga *et al*., 2012).

This study highlights the importance of extraction methods effects on extract composition and overall properties. While extract A is a less-refined *Sceletium tortuosum* extract, it appears to exert more beneficial effects at a broad range of doses, which is promising in the setting of non-communicable inflammatory disease. Although extract B does possess properties that may assist in prevention of chronic inflammation-associated neurodegeneration, where oxidative stress is both an etiological and comorbid factor, its highly-refined nature resulted in a much more potent antioxidant product, which needs to be carefully administered at specific, low doses to achieve desired effects whilst also minimising undesired pro-oxidant damage.

# Chapter 4 – Experiment II

In this chapter, data are again presented in manuscript format, as submitted for publication to *Journal of Affective Disorders* (Impact Factor 3.43). However, in addition, since a large portion of effort was invested in development of the model, I also present pilot data illustrating the process followed in setting up the platelet model elsewhere (refer to Appendix F).

# Diagnosis and *in vitro* pharmacological testing in acute and chronic anxiety: illustrated relevance of the platelet serotonergic system

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## Abstract

*Background:* Anxiety is characterised by disorders in serotonin neurotransmission. Pro-inflammatory cytokines are also responsible for undesired modulation of serotonin signalling, metabolism, and receptor expression. Platelets possess structural proteins that are identical to serotonergic neurons. These similarities led to the hypothesis that platelets could be employed in diagnosis and therapeutic monitoring of anxiety.

*Methods:* Modulators of the serotonergic system – a selective serotonin reuptake inhibitor and a monoamine releasing agent (MRA) – were used to investigate the platelet model in anxiety. Isolated platelets from anxious and non-anxious subjects were exposed to a known activator (calcium ionophore A23187) and each modulator for 15 minutes. Cytokine secretion and changes in platelet serotonergic system activity were assessed.

*Results:* Basal platelet serotonin levels in individuals exhibiting state/trait anxiety were lower when compared to no-anxiety controls ($p<0.05$), while platelet activation state was increased (significantly lower CD63 ($p<0.05$)). Serotonin transporter expression was down-regulated in state anxiety in response to citalopram treatment, validating the platelet model. Platelet serotonergic activity reflected differences in modulation of the system in terms of serotonin release and reuptake. The MRA – Trimesemine™ – was shown to have anti-inflammatory effect via modulation of pro- and anti-inflammatory cytokine secretion.

*Conclusions*: This study has highlighted key differences between anxious and non-anxious individuals, and between state and trait anxiety, in terms of serotonergic system parameters and inflammatory responses, suggesting diagnostic potential for the platelet model. Changes in platelet serotonin stores, release and reuptake dynamics, and cytokine production indicate that this model is sufficiently sensitive for therapeutic monitoring in individuals with anxiety.

## 4.1 Background

Anxiety is currently one of the most common mental disorders, characterised by high levels of emotional distress and functional impairment (Baxter *et al*., 2013; Leal *et al*., 2017). Two complementary concepts of anxiety exist: state anxiety, which refers to psychological and physiological reactions resulting from isolated adverse situations, and trait anxiety, which refers to an individual's personal tendency to present state anxiety chronically (Leal *et al*., 2017).

This phenomenon of experiencing internal psychological stress in the absence of external stressors results in chronic activation of the stress response pathways, the products of which often have detrimental consequences for an individual's wellbeing. The extent and duration of stress-induced alterations in glucocorticoids and neurotransmitters have substantial effects on overall psychological health, in addition to facilitating a relative pro-inflammatory status (Basu and Dasgupta, 2000; Dhabhar, 2009). It is here where individual differences become relevant while studying human models, as stress perception and coping methods can have significant effects on the kinetics, peak levels and duration of elevated stress hormones and cytokines in circulation (Dhabhar, 2009).

Enduring psychological stress, such as in anxiety, is characterised by disorders in serotonin, or 5-hydroxytryptamine (5-HT), neurotransmission. It is hypothesised that immune activation may be related to these transmission defects, as pro-inflammatory cytokines are responsible for the lowered activity of presynaptic 5-HT neurons, alterations in 5-HT re-uptake from the synaptic cleft and changes in post-synaptic cleft 5-HT receptors (Maes *et al*., 1997). Similarly, 5-HT is known to modulate several leukocyte functions (Schiepers *et al*., 2005).

Apart from its obvious diagnostic relevance, central serotonergic signalling is of great importance for monitoring the effects of anxiolytic and antidepressant medicines. Interestingly, expression of the SERT, responsible for the maintenance of serotonergic function by regulating 5-HT sequestration into cells, has been characterized in a range of cell types outside of the central compartment (Dürk *et al*., 2005; Hoirisch-Clapauch *et al*., 2014; Scharinger *et al*., 2014). Most importantly, platelets appear to possess SERT proteins that are identical to those in the central nervous system (Lesch *et al*., 1993).

Platelets also possess small storage and secretion granules – δ-granules – that resemble dense-core vesicles in neurons, in that they contain 5-HT, which is taken up and concentrated from the extracellular environment (Jedlitschky *et al*., 2012). The release of 5-HT by platelets promotes platelet functions via the serotonin receptor, $5\text{-HT}_{2A}$. When platelets adhere and aggregate at a site of vessel injury, 5-HT is secreted, which directly accelerates platelet aggregation and potentiates the response of platelets to other agonists (Hoirisch-Clapauch *et al*., 2014). Human platelets have been used as a model to study 5-HT release dynamics, due to

these key similarities with serotonergic nerve terminals (Alvarez *et al*., 1999; Jedlitschky *et al*., 2012; Mumford *et al*., 2015).

In the context of the immune system, secretion of the anti-inflammatory cytokine, IL-10, has been shown to be significantly inhibited in patients with anxiety, while increases in pro-inflammatory cytokines such as TNF-α and IL-17 are associated with general anxiety patients (Furtado and Katzman, 2015a). Platelets are not only storage sites for bioactive molecules, but also generate mediators such as thromboxane $A_2$, and produce both pro-inflammatory and anti-inflammatory molecules, the most abundant of which are TNF-α, IL-8, platelet factor-4, MCP-1 and IL-10 (Gawaz *et al*., 2005; Nurden, 2011). In addition, platelets possess several extranuclear mechanisms that translate mRNA into proteins, and as a result can produce IL-1β and tissue factors (Smyth *et al*., 2009).

Although laboratory assays that measure the content and release of granules were introduced over 40 years ago (Gresele *et al*., 2014), incorporation of these into clinical diagnostic practice remains inconsistent. However, the similarities between the platelet and neural serotonergic systems led us to hypothesise that platelets could be employed both in diagnosis and therapeutic monitoring in anxiety.

To test our hypothesis, we selected two modulators of the serotonergic system with which to investigate the relevance of the platelet model in the context of anxiety. Firstly, citalopram was selected as reference SSRI (Matthäus *et al*., 2016). Secondly, we identified a commercially available plant extract (Trimesemine™) with proven anxiolytic (Bennett and Smith, 2018; Smith, 2011; Swart and Smith, 2016) and monoamine-releasing (Coetzee *et al*., 2016) properties. Our choice of this product was based on the fact that it does not seem to elicit the undesired side-effects often reported for other MRAs, such as methamphetamine (Connor, 2004; Verrico *et al*., 2007).

## 4.2 Methods & Materials

### 4.2.1 Ethical considerations

This study was approved by Stellenbosch University's Health Research Ethics Committee (Study reference: SU-HSD-003976) and was carried out in accordance with the guidelines of the South African Medical Research Council.

### 4.2.2 Subject recruitment and sample collection

Normally healthy male and female individuals between the ages of 21 – 35 years old were recruited for participation in this study. A full blood count was performed on whole blood from all participants, using automated analysis (Celldyne 3700CS, Abbott Diagnostics) to ensure all relevant blood parameters were satisfactory. In addition, a lifestyle questionnaire was completed by each participant to confirm overall health status, and to identify potential confounders that may affect inflammatory status and platelet function. This was followed by assessment of platelet parameters (international normalized ratio (INR) and activated partial thromboplastin time (APTT)), performed by automated analysis by commercial pathology laboratory (PathCare, Stellenbosch, South Africa) to determine coagulability status to reduce any confounding effects in the context of 5-HT release due to differences in platelet activation status.

Only individuals with no recent illness (within 30 days) and no chronic inflammatory disorder were allowed into the study. All participants were also required to refrain from supplement use and alcohol or other drug consumption for at least one week prior to blood sampling.

Participants completed a State-Trait Anxiety Index (STAI) questionnaire (Appendix E), a widely used measure of anxiety status (Julian, 2011), at the time of blood sampling (±20ml, sodium citrate anti-coagulated) for the platelet assay. Participants were divided into categories of state and/or trait anxiety, according to their STAI scores (n=6 trait anxious, n=7 non-trait anxious, n=5 state anxious and n=8 non-state anxious individuals).

Each questionnaire (for either state or trait anxiety diagnosis) contains 20 statements that participants are required to rate on a 4-point scale (e.g. from "Not At All" to "Very Much So"). These statements include: "I am worried" and "I feel content" for the state anxiety form and "I feel nervous and restless" and "I am a steady person" for trait anxiety analysis. The STAI is appropriate for those who have at least a sixth-grade reading level. A score of 39 and above on each form indicates the presence of either state or trait anxiety, or both.

### 4.2.3 Platelet assay

Isolated platelets were exposed to a known activator, calcium ionophore A23187, TRI and citalopram for a period of 15 minutes at room temperature to assess changes in serotonergic system activity and platelet cytokine secretion. Vehicle (PBS) only was added to all control wells.

Following treatment intervention, platelets were centrifuged (400xg, 15 minutes, room temperature, no brake). The resulting plasma supernatant was aspirated and stored at -80°C for subsequent batch analysis of secreted cytokines, while the platelet samples were prepared for flow cytometry analysis of expression levels of selected parameters.

#### *4.2.3.1 Platelet isolation*

Since platelets are, by nature, highly sensitive to stimuli and naturally prone to activation, it was important to optimise the isolation protocol to prevent spontaneous activation due to changes in temperature, as well as handling and centrifugation forces. Some available protocols suggest the use of prostaglandin E1 to inhibit platelet activation and aggregation during isolation; however, this was not possible in the current study, since platelet activation in response to treatment was of interest. It was important that a low percentage of platelets remained inactivated before treatment intervention to effectively quantify changes. The optimisation process can be seen in Appendix F.

Platelet count in whole blood was determined using automated analysis as mentioned. Platelets were then isolated in a series of wash and centrifugation steps. Briefly, whole blood was centrifuged (400xg, 15 minutes, room temperature, no brake), and the resulting plasma was gently aspirated, avoiding white blood cell contamination, and pooled in a new, sterile centrifuge tube.

Plasma was centrifuged (400xg, 15 minutes, room temperature, no brake) to allow for separation into platelet-rich plasma (PRP) (lower two-thirds) and platelet-poor plasma (PPP) (upper one-third). The PPP layer was gently aspirated and stored at -80°C for later batch analysis.

An aliquot of the remaining PRP was analysed to determine final platelet concentration. The PRP was diluted with PBS to achieve a final concentration of $1x10^8$ platelets/ml. 1ml of platelet suspension was placed into each of four sterile 15ml centrifuge tubes.

#### *4.2.3.2 Treatment intervention*

##### 4.2.3.2.1 Calcium ionophore A23187

Platelets were treated with a final concentration of 1nmol/ml of a calcium ionophore, as per convention in the literature (Gobbi *et al*., 2003). A working stock of 100nmol/L was made by dissolving calcium ionophore A23187 (Sigma-Aldrich, cat# C7522-5MG) in 99.7% dimethyl sulfoxide (DMSO) and diluting with PBS, as per manufacturer's instructions. 10µl of working stock was added to the activated control group of platelets.

##### 4.2.3.2.2 *Sceletium tortuosum* extract (Trimesemine™)

Trimesemine™ (TRI, batch number: DV SCITRI:E 591/016, Verve Dynamics Ltd, Somerset West, South Africa) is a commercially available extract prepared from a variety of *Sceletium tortuosum* that has been selectively propagated to achieve high mesembrine content (68.4% of total alkaloidal content). Extract characteristics are presented in Figure 3.1 and Table 3.1.

Platelets were treated with a final TRI concentration of 0.01mg/ml, as this concentration of the extract has been most consistently reported to have beneficial effects in other models (Bennett and Smith, 2018; Coetzee *et al*., 2016; Swart and Smith, 2016). A working stock of 1mg/ml was made by combining isolate powder and pre-warmed PBS. The mixture was vortexed for two minutes and sterile-filtered using a 0.22µm syringe filter. 10µl of working stock was added to the treatment group of platelets.

#### 4.2.3.2.3 Citalopram

Platelets were treated with a final citalopram concentration of 10µmol/L per $1x10^6$ platelets (Coetzee *et al*., 2016). A working stock solution of 1mg/ml (2.45mM) was made by dissolving citalopram powder (Sigma-Aldrich, cat# C7861) in methanol and diluting to the desired concentration with PBS, as per manufacturer's instructions. 5µl of working stock per $1x10^6$ platelets was added to the SSRI control group.

### 4.2.4 Flow cytometric analysis

Platelet pellets were resuspended in 500µl 1X BD FACS-lyse solution and incubated for 10 minutes. Platelets were then washed with PBS and centrifuged (800xg, 20 minutes, 4°C), the supernatant removed and discarded, and platelets resuspended in 500µl 0.1% saponin for 10 minutes (duration and concentration of both FACS-lyse and saponin treatments were optimized in a pilot study). Following the permeabilization, platelets were washed with PBS and centrifuged (800xg, 20 minutes, 4°C), before being finally resuspended in 100µl PBS with 3% FBS.

Primary antibodies for SERT (goat anti-human) and 5-HT (rat anti-human) were added, and platelets were incubated in the dark for one hour at 4°C. Subsequently, platelets were washed with PBS (with 3% FBS and 0.1% saponin) and centrifuged (400xg, 5 minutes, 4°C). Platelets were stained with secondary antibodies (Alexa Fluor™ 488-conjugated donkey anti-goat immunoglobulin-G (IgG) and Alexa Fluor™ 647-conjugated donkey anti-rat IgG), and again incubated in the dark for 1 hour at 4°C.

Following an additional wash step, platelets were stained with conjugated antibodies for VMAT-2 (Alexa Fluor™ 405) (associated with 5-HT release), CD63 (BD Horizon™ PE-CF594) (marker of dense granules, which contain 5-HT) and CD41a (BD Horizon™ PE-Cy7) (general platelet activation marker) and incubated in the dark for 30 minutes at 4°C. A final wash step was performed, after which platelets were resuspended in 1% paraformaldehyde and assessed by flow cytometric analysis (BD FACSAriaII with *BD FACSDiva v8.1 Software)*. CD41a was used as a platelet gating marker (Figure 4.1), as it is expressed by platelets and megakaryocytes only (Gobbi *et al*., 2003), and therefore data for this parameter is excluded from the results.

For instrument setup, single stain and FMO strategies, and gating strategies for all parameters employed in this study, see Appendix G.

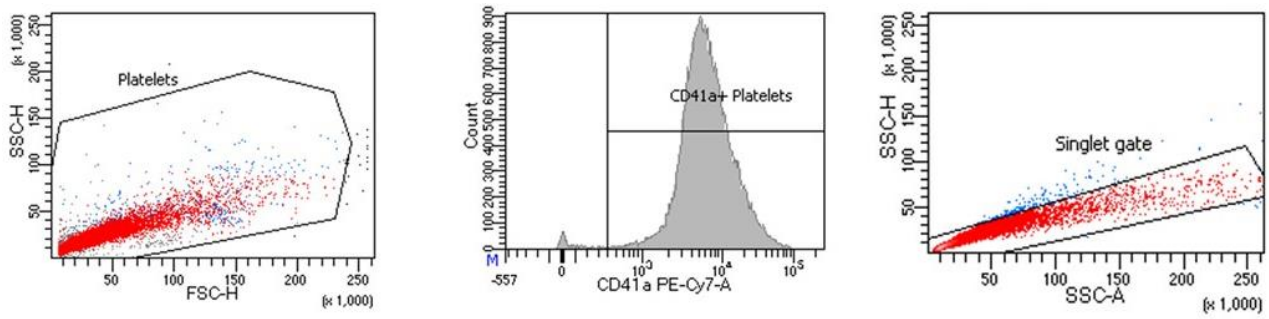


*Figure 4.1: Representative flow cytometry scatter plots and histogram illustrating platelet gating strategy based on CD41a expression*

### 4.2.5 Cytokine quantification

Preserved platelet culture supernatants were analysed using commercial Magnetic Bead Panel assays (custom-designed Milliplex MAP Human Soluble Cytokine Receptor Panels, Merck Millipore) for concentrations of IL-1β, IL-6, IL-8, IL-10, neutrophil-activating peptide-2 (NAP2), TNF-α, MCP-1 and soluble CD40-ligand (sCD40L) (Appendix B). The fluorescent signals were analysed using a Bio-Rad Bio-Plex® 200 instrument with *Bio-Plex Manager v6.1* software.

Quantification of cytokine concentrations was performed based on a standard curve, derived from linear dilution of the manufacturer-supplied cytokine standards. The detection limit was 0.8pg/ml for IL-1β, 0.9pg/ml for IL-6, 0.4pg/ml for IL-8, 1.1pg/ml for IL-10, 11.2pg/ml for NAP2, 0.7pg/ml for TNF-α, 1.6pg/ml for MCP-1, and 5.1pg/ml for sCD40L. Levels of secretion for IL-1β and IL-6 were extremely low in most cases. Thus, this data was excluded from statistical analysis.

### 4.2.6 Statistical analysis

Results in graphs are presented as means, and standard error of the means (SEMs). Normality of data distribution was assessed, followed by non-parametric 2-way ANOVA and LSD *post hoc* tests. In cases where Levene's Test for Homogeneity of Variances rejected the null hypothesis, the Games-Howell *post hoc* test was used (*Statistica v13.2*). Results in tables are presented as means ± standard deviations.

## 4.3 Results

Basic participant characteristics and clotting profiles are presented in Table 4.1. All parameters were within the expected normal ranges for all individuals (n=13, nine males and four females).

Table 4.1: Participant basic characteristics at blood sampling, as well as basic haematology profiles

| | Expected normal range | Mean ± standard deviation (n=13) |
|---|---|---|
| Age (years) | | 25 ± 2.75 |
| Height (cm) | | 173.33 ± 8.55 |
| Weight (kg) | | 74.16 ± 10.87 |
| Body mass index | 18.5 – 24.9 | 24.81 ± 2.15 |
| *Baseline clotting parameters:* | | |
| INR | 0.9 – 1.3 | 1.07 ± 0.08 |
| APTT | 25.4 – 38.4 | 31.32 ± 2.80 |
| *Baseline whole blood parameters:* | | |
| Leukocyte count ($10^9$ cells/L) | 4.00 – 10.25 | 6.07 ± 1.40 |
| Red blood cell count ($10^{12}$ cells/L) | 3.70 – 6.18 | 4.83 ± 0.36 |
| Platelet count ($10^9$ cells/L) | 145 – 400 | 239.38 ± 58.69 |
| Haematocrit (%) | 36 – 54 | 41.16 ± 4.14 |

According to anxiety profile of the study population, state and trait anxiety scores were correlated in this population as expected ($p<0.001$) (Figure 4.2A). Six individuals fulfilled clinical criteria for trait anxiety and five for state anxiety (including four exhibiting both trait and state anxiety) (Figure 4.2B).

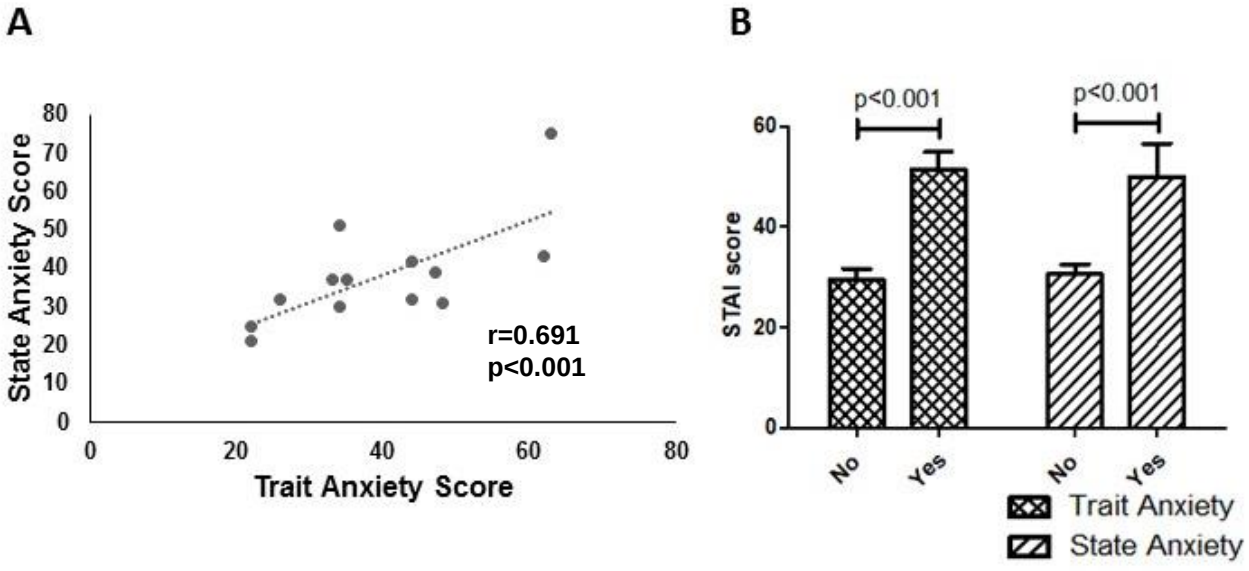


*Figure 4.2: (A) State-trait anxiety correlation and (B) quantification of participants with/without either state or trait anxiety, as determined by STAI scoring.*

For all individuals exhibiting anxiety, either state or trait anxiety, platelet intracellular 5-HT levels were significantly lower when compared to no-anxiety controls (Figure 4.3A and B), with no additional effect apparent for either treatment. In no-anxiety controls, both TRI and citalopram decreased 5-HT levels significantly.

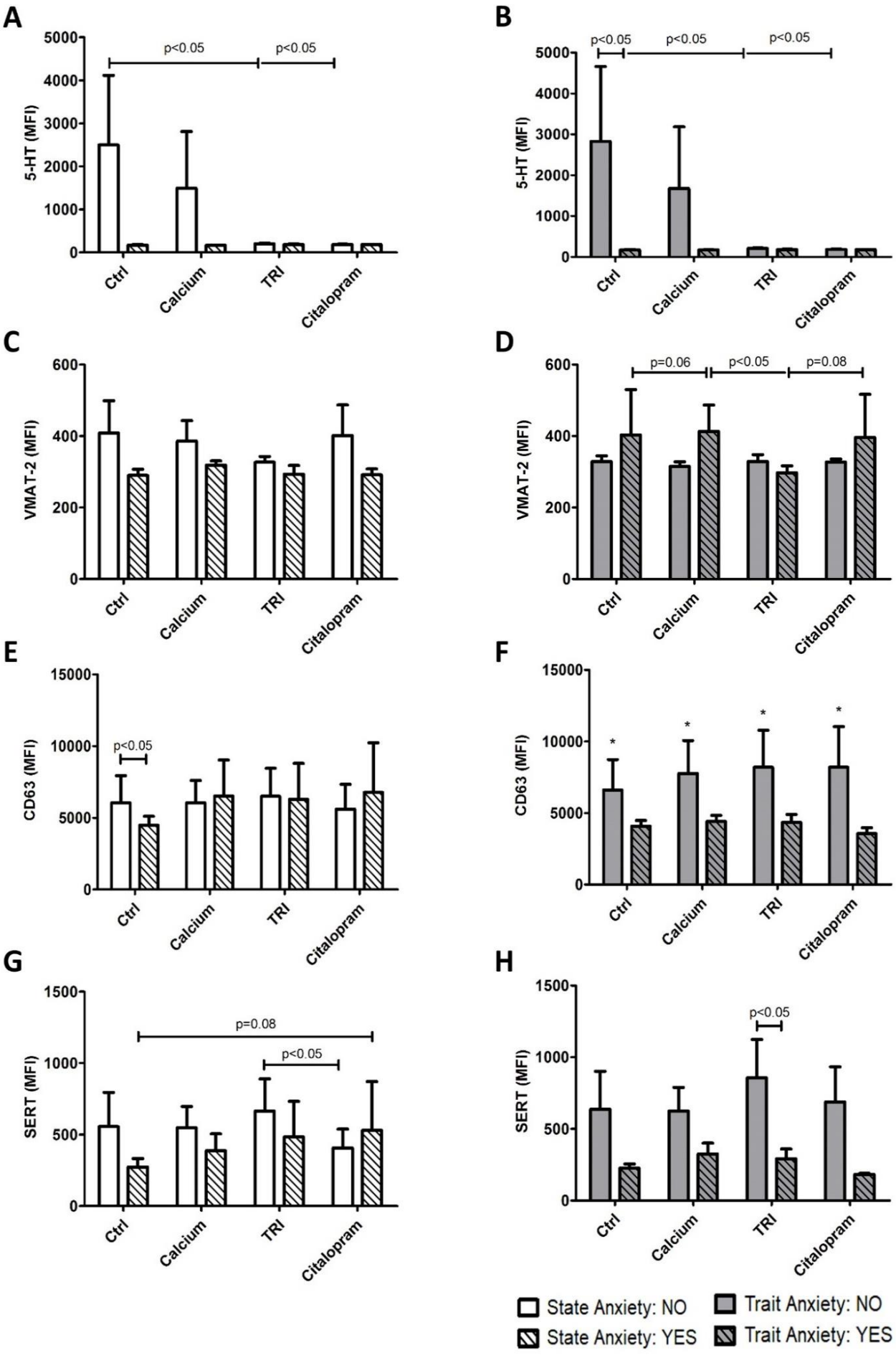

*Figure 4.3: (A) Platelet intracellular 5-HT content in samples obtained from individuals with/without state anxiety and (B) with/without trait anxiety. (C-F) Platelet parameters indicative of 5-HT storage and/or release in individuals with/without state or trait anxiety respectively: membrane VMAT-2 and CD63. (G and H) Platelet membrane-bound SERT, indicative of 5-HT re-uptake potential, in individuals with/without state or trait anxiety. *p<0.00001*

For VMAT-2, neither state nor trait anxiety seemed to affect expression levels (Figure 4.3C and D). However, TRI significantly reduced VMAT-2 expression in individuals exhibiting trait anxiety, when compared to other conditions. In contrast, both state and trait anxiety significantly decreased CD63 expression on platelets (Figure 4.3E and F; main effect of trait anxiety, $p<0.00001$). This parameter did not seem to be affected by either treatment.

In terms of 5-HT re-uptake, SERT expression showed a weak tendency to be lower for trait anxiety (ANOVA main effect of trait anxiety, $p=0.09$) but not state anxiety when compared to controls (Figure 4.3G and H). In individuals with state anxiety, citalopram showed a tendency for increasing SERT expression. In individuals with no state anxiety, SERT expression was higher after treatment with TRI when compared to treatment with citalopram (Figure 4.3G). In line with this, the decrease in SERT expression as result of trait anxiety only reached significance in the TRI treatment condition.

Table 4.2 shows the concentrations (pg/ml) of each cytokine measured for this study. Due to large inter-subject variation, each subject was standardised to their own untreated control, with cytokine concentrations expressed as a percentage of their control conditions (Figure 4.5 and Figure 4.6). Due to the variability between individuals, we also decided to not exclude values on the lower detectable limit, as these gave valuable insight into the effects of anxiety on subject responsiveness to treatment interventions.

Table 4.2: Platelet supernatant cytokine concentrations following treatment intervention

| | Cytokine concentration (pg/ml) (mean ± standard deviation, n=13) | | | | | |
|---|---|---|---|---|---|---|
| | TNF-α | IL-8 | MCP-1 | IL-10 | sCD40L | NAP2 |
| Control | 11.07±5.65 | 5.20±5.91 | 185.20±43.57 | 6.76±9.09 | 115.77±42.59 | 637154.28± 197121.28 |
| Calcium ionophore | 11.79±5.73 | 5.12±5.46 | 186.31±38.13 | 6.85±9.08 | 134.32±58.66 | 693961.85± 168077.96 |
| Trimesemine | 9.50±6.16 | 4.85±6.10 | 168.05±50.69 | 6.05±9.21 | 108.16±47.52 | 596910.98± 150128.42 |
| Citalopram | 11.08±5.67 | 5.75±6.10 | 187.12±45.76 | 6.37±10.02 | 111.75±35.23 | 577337.05± 150246.34 |

The variability between participants was further exacerbated by the presence or absence of state and/or trait anxiety. An example of this can be seen when looking at the changes in absolute concentrations of MCP-1 (Figure 4.4A-D) in the supernatant, following treatment intervention. This interpersonal variability due to anxiety type further supported the expression of each cytokine concentration as a percentage of its control conditions.

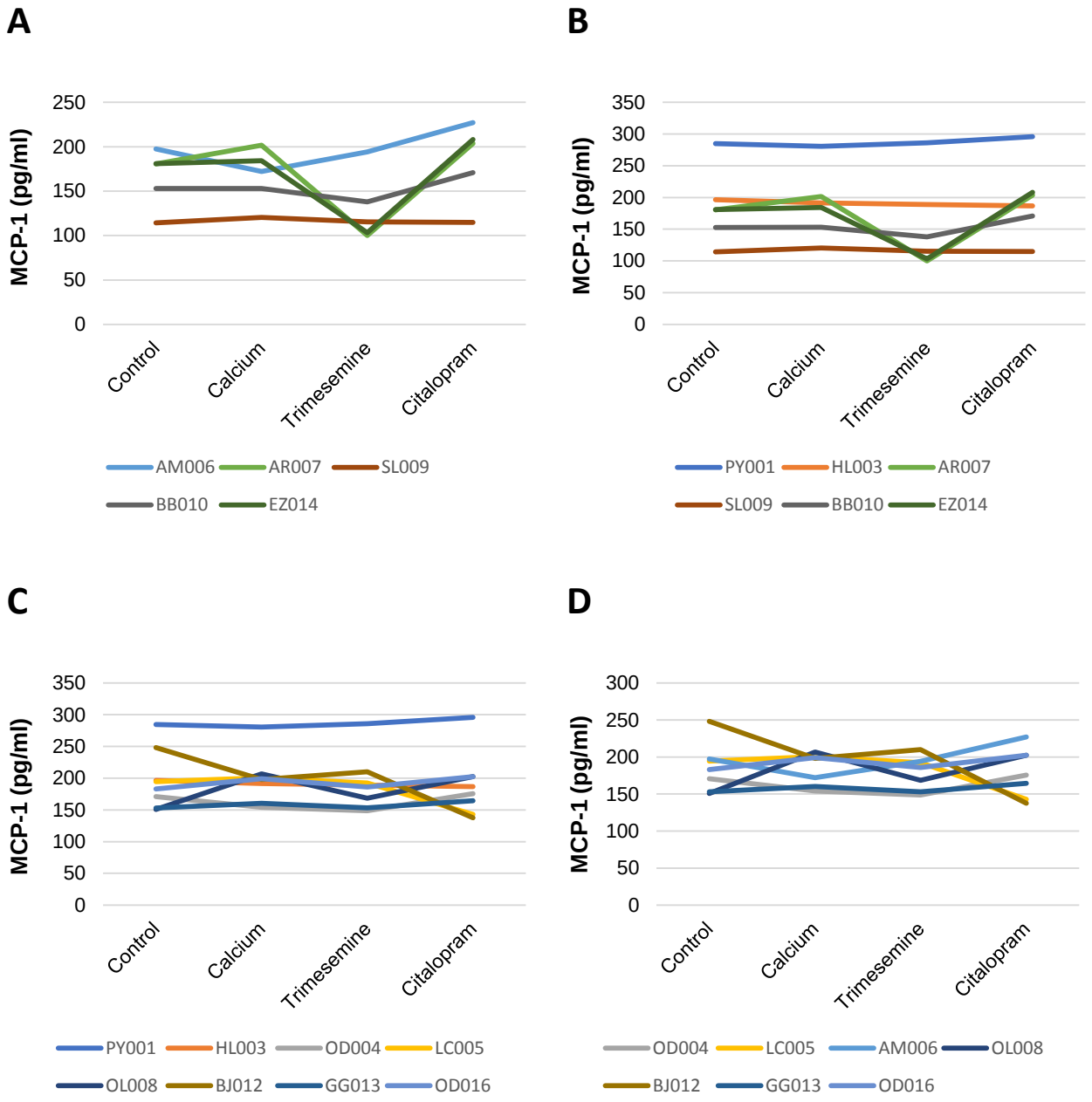


*Figure 4.4: Absolute concentration of MCP-1 in platelet supernatant following treatment intervention, in (A) state anxious individuals, (B) trait anxious individuals, (C) non-state anxious individuals and (D) non-trait anxious individuals*

In terms of pro-inflammatory cytokines commonly assessed in the neuroimmunology literature – TNF-a, IL-8 and MCP-1 – TRI generally seemed to have a suppressive effect on platelet pro-inflammatory cytokine release (Figure 4.5A-F). In line with this, there was a tendency for higher IL-10 in the TRI treatment condition when

compared to citalopram (Figure 4.5G and H) in the state anxiety group. In contrast, citalopram did not appear to have any effect on these parameters.

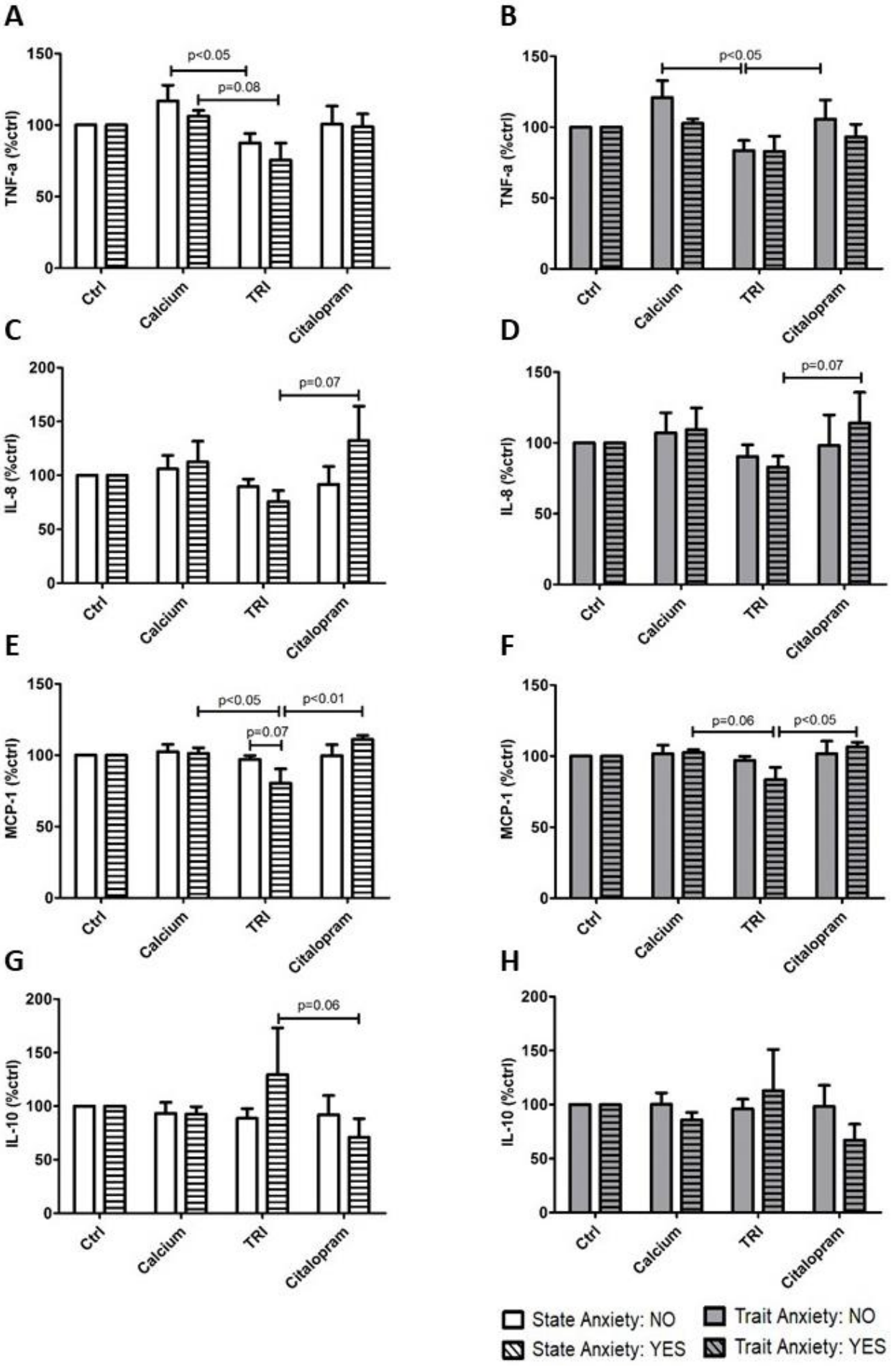

*Figure 4.5: Pro-inflammatory cytokine secretion assessed in supernatants of platelet primary cultures treated with either a calcium ionophore, TRI or citalopram, in both (A,C,E) state and (B,D,F) trait anxiety. Anti-inflammatory cytokine secretion assessed in supernatants of platelet primary cultures treated with either a calcium ionophore, TRI or citalopram, in both (G) and (H) trait anxiety*

In this model, secretion levels of platelet-specific markers of activation – sCD40L and NAP2 – were in line with decreased serotonergic system-specific activation (CD63) as result of trait anxiety but did not appear similarly sensitive to reflect anti-inflammatory effects of TRI (Figure 4.6A-D).

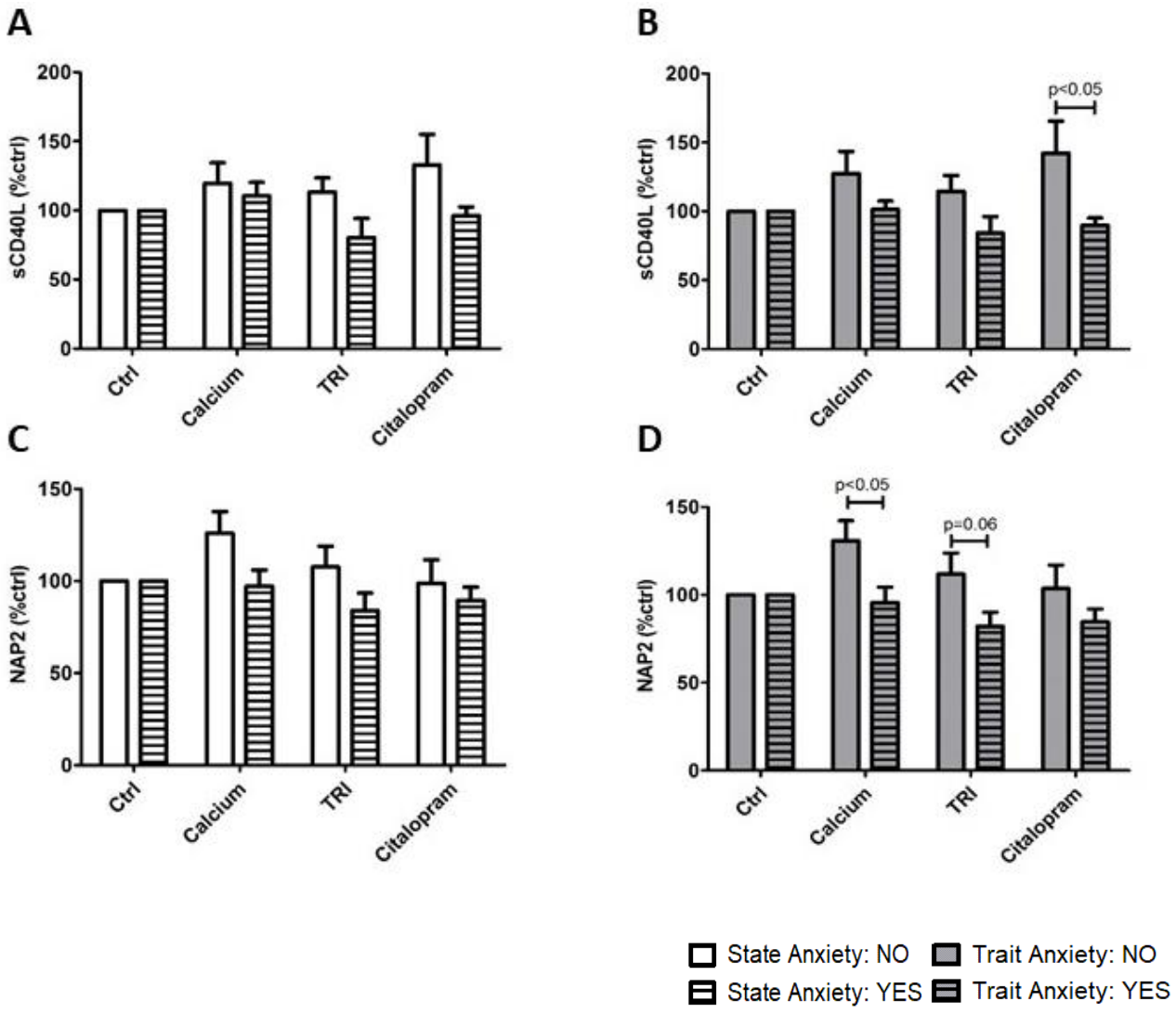


*Figure 4.6: Platelet-specific markers of inflammatory activation assessed in supernatants of platelet primary cultures treated with either a calcium ionophore, TRI or citalopram, in both (A,C) and (B,D) trait anxiety*

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## 4.4 Discussion

As agreement with the literature on neuronal SERT (Matthäus *et al*., 2016; White *et al*., 2005), platelet SERT expression was down-regulated in the state anxiety model, in response to citalopram treatment. This validates platelets as a suitable model of the central serotonergic system in this setting. In addition, results reported here in platelets are in line with those reported after similar studies performed on 5-HT-secreting neural cells (Coetzee *et al*., 2016; Jaiswal *et al*., 2015). Furthermore, in the absence of anxiety, changes in intraplatelet 5-HT were evident between the control and treatment groups, further supporting the use of this model to investigate 5-HT release using platelets from normally healthy individuals.

In the setting of anxiety, this study has highlighted key differences not only between anxious and non-anxious individuals, but also between anxiety types, in terms of serotonergic system parameters as well as inflammatory responses. Since the physiological response to anxiety involves the release of 5-HT from serotonergic neurons and platelet stores (Graeff *et al*., 1996), it is understandable that intraplatelet levels of 5-HT and VMAT-2 are already low in the control groups of both state- and trait-anxious individuals (Figure 4.3A and B). These findings are supported by previous studies that showed low levels of 5-HT in the ventral striatum of rats with anxiety (Schwarting *et al*., 1998; Sommer *et al*., 2001).

However, the differences between anxious and non-anxious individuals in terms of intraplatelet 5-HT are staggering, with platelets from non-anxious individuals containing ±15 times more 5-HT than anxious (both state and trait) individuals under basal conditions. This corresponds with platelet CD63 expression (in trait anxiety). CD63 is indicative of the presence of δ-granules, the storage site for platelet 5-HT. The fact that the effect of anxiety on CD63 is only evident in the trait group, suggests that while 5-HT levels cannot distinguish between acute and chronic anxiety states, CD63 as marker is less sensitive to acute anxiety.

The implication here is that while 5-HT release is a relatively acute process, the downregulation of dense granule content in platelets occur more gradually. Nevertheless, in terms of the applicability of the platelet model, the significantly lower intraplatelet 5-HT content and CD63 expression are indicative of depletion of this neurotransmitter in anxiety states, albeit at different rates, to the point where the effects of any treatment cannot be observed. This limitation of the model is probably a result of the fact that platelets cannot synthesise 5-HT (Hoirisch-Clapauch *et al*., 2014). An option to investigate may be to supplement the culture media with 5-HT to replenish intraplatelet 5-HT stores – although this may interfere with assessments of SSRI function, it may be beneficial for the study of 5-HT release and the effect of pharmaceuticals on this process.

Somewhat similar to the CD63 data, the study of membrane VMAT-2 expression also elucidated significant differences between state and trait anxiety. While VMAT-2 levels appeared unaffected by state anxiety, trait anxiety was associated with a decrease in membrane VMAT-2 expression in response to calcium ionophore

A23187 activation, suggesting an imbalance between 5-HT release and reuptake in individuals with trait anxiety (Reimold *et al*., 2008; Schwarting *et al*., 1998). Interestingly, after TRI treatment VMAT-2 expression decreased almost 1.5-fold compared to the non-treated group upon activation, sensitively reflecting the MRA function of TRI.

Together, these findings provide important information on the use of the platelet model for the study of 5-HT secretion. Firstly, the platelet model may be an accurate tool for differential diagnosis and monitoring of anxiety once a reference range for platelet levels of the parameter panel has been established. Secondly, platelets from non-anxious donors could be used in drug development studies, to provide sufficient capacity to reflect therapeutic effects in platelet 5-HT level.

In further support of our choice of MRA substance, *Sceletium tortuosum* has, until recently, been regarded primarily as an SSRI (Gericke and Viljoen, 2008; Terburg *et al*., 2013). However, this concept was challenged by findings from a recent study on TRI, which found that the high-mesembrine *Sceletium* extract had a greater up-regulatory effect on VMAT-2 expression in comparison to citalopram treatment (Coetzee *et al*., 2016). Since VMAT-2 is known to facilitate monoamine storage as well as release (Hoirisch-Clapauch *et al*., 2014), these findings suggested that TRI may act more so as an MRA, and possesses SSRI activity only as a secondary effect. Current data confirms this interpretation, specifically in the non-trait anxiety group, where VMAT-2 expression after TRI exposure was significantly lower than in the calcium ionophore A23187-activated group and tended to be lower than in platelets exposed to citalopram.

Furthermore, when considering platelet contribution to the inflammatory response characterising anxiety states, it was apparent that TRI exerts the most favourable effects in terms of limiting pro-inflammatory cytokine production, especially when compared to citalopram, which has been found to increase levels of IL-1β and TNF-α (Furtado and Katzman, 2015b; Munzer *et al*., 2013). This is in line with our previous work (Bennett *et al*., manuscript under review), where human astrocyte pro-inflammatory cytokine production was significantly limited by both 0.01mg/ml and 1mg/ml TRI doses in the setting of acute endotoxin stimulation (*E.coli* LPS). These findings indicate that the platelet model is sensitive enough to illustrate inflammatory side-effects that may be associated with certain anxiolytic and anti-depressant treatments.

In addition, TRI has also shown to increase secretion of IL-10 in both the current platelet model and in human astrocytes. Since both anxiety and depression have been shown to stimulate the secretion of pro-inflammatory cytokines (Anisman and Merali, 2002; Kiecolt-Glaser *et al*., 2015; Miller *et al*., 2009; Schiepers *et al*., 2005), this inflammation-limiting effect linked to TRI exposure is an added benefit, which may, at least in part, contribute to its better safety profile in comparison to other MRAs.

Finally, investigation of platelet-specific inflammatory activation, in terms of sCD40L and NAP2 expression, did not reflect differences between control or anxiety states. Similarly, neither sCD40L, nor NAP2 was

affected by either treatment employed. Thus, in the context of anxiety-related inflammation, these are probably less useful markers.

# Chapter 5 – Final Conclusions and Directions for Future Research

Improving lifestyle stress exposure and making responsible choices about one's health and wellbeing are vital in elevating overall health status. Managing continual stress exposure requires the ability to recognize stressors and respond constructively, limiting behaviours that exacerbate the strain on physiological stress-response mechanisms. However, it is not always easy to avoid chronic stress, especially in the fast-paced, success-driven world today. Therefore, research into potential therapeutic interventions for consequences of the modern-day lifestyle is of great importance.

From current data it is clear that the *Sceletium tortuosum* extracts tested have potential in the preventative medicine niche, specifically in terms of limiting inflammation and/or oxidative stress to achieve a rate-limiting effect on chronic inflammatory disease development. However, the effects of *Sceletium* extracts and/or their isolated pure alkaloids on endocrine-immune interaction need to be further elucidated, to enable optimisation of the use of this indigenous product in the setting of chronic lifestyle diseases, such as type II and type III diabetes.

Distinct, bidirectional links exist between enduring psychological stress, chronic activation of the inflammatory response, and the development of non-communicable diseases. The severity of physiological maladaptations in response to recurring lifestyle stress or in response to traumatic events are clear in our platelet assay results, where significant differences are evident in acute and chronic anxiety, in terms of response to commercial treatments. This is confirmed by Ross *et al*. (2017), whose extensive review on chronic stress in anxiety and depression concluded that patients with comorbid depression and anxiety are less likely to respond effectively, if at all, to currently approved medications.

Centrally, anxiety and depression are both a cause and result of 5-HT depletion in serotonergic neurons, and decreased receptor and reuptake transporter density. From the platelet model, we could confirm that 5-HT hyperresponsiveness is a hallmark maladaptation in the setting of repeated stress-response stimulation, but not in acute stimulation, with low intraplatelet 5-HT and increased platelet activation in trait anxiety.

The significant changes in platelet 5-HT stores, release and reuptake dynamics, and major cytokine production indicate that this model is useful in the study of 5-HT-mediated inflammation, specifically in the context of anxiety. Our platelet model has also indicated how differing degrees of mental health contribute to challenges in diagnosis and timely treatment. 5-HT reuptake and release assays would shed more light on this, where intraplatelet stores can be replenished before treatment exposure, to observe changes in response to treatments in anxious individuals.

Assessment of changes in expression of platelet SERT and VMAT-2 following treatment exposure, performed at a series of time points, would give greater insight into the mechanisms of action behind 5-HT release dynamics. For example, since platelets cannot synthesise 5-HT, continual 5-HT release is facilitated by

monoamine reuptake via SERT. Thus, if prolonged exposure to the mild and intended SSRI effects of TRI and citalopram, respectively, may result in VMAT-2 downregulation over time, but this would not be immediately evident.

Further elucidation of *Sceletium tortuosum's* effects on cytokine secretion may be achieved through investigation of the *in vivo* effects of *Sceletium* supplementation on immune cells, which may provide information regarding biological factors that may act in synergism or antagonism with this natural product. This setting may also lend itself to examination of relative pro- and anti-inflammatory cytokine peaks and half-lives, through the study of multiple time points following treatment intervention.

# Chapter 6 – References

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# Chapter 7 – Appendices

## Appendix A – Immunomodulatory effects of *Sceletium tortuosum* elucidated *in vitro*: Implications for chronic disease

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## Abstract

*Ethnopharmacological relevance*: *Sceletium tortuosum*, among other *Sceletium* species, was traditionally used by the Khoisan people of Southern Africa for relief of pain-related ailments. However, the commercial availability of this supplement has greatly expanded due to anecdotal claims of its mood-elevating and anxiolytic properties. Unrelated research has elucidated a significant link between cytokines and the mediation of depression. Therefore, the effect of *Sceletium* supplementation on immune cell functionality is of interest, since the efficacy of potential depression treatments could, at least in part, rely on downregulation of pro-inflammatory signalling.

*Aim of the study*: The current study evaluated the immunomodulatory effects of a *Sceletium* extract, both basally and in the context of acute endotoxin stimulation.

*Materials and Methods*: Primary human monocytes were supplemented with either a 0.01mg/ml or 1mg/ml *Sceletium* extract dose, with or without E. coli LPS stimulation *in vitro*, for 24 hours. Mitochondrial viability, as an indirect measure of cytotoxicity, and cytokine release in response to the treatment intervention were assessed.

*Results*: *Sceletium* extract treatment was associated with increased mitochondrial viability, as well as up-regulated IL-10 release under basal conditions. LPS exposure significantly decreased mitochondrial viability, but this was prevented completely under *Sceletium*-treated conditions. The acute inflammatory response to LPS stimulation was not negatively affected. *Sceletium* treatment conferred most significant effects at a dose of 0.01mg/ml.

*Conclusions*: *Sceletium* exerts significant cytoprotective effects in the setting of endotoxin stimulation. Cytokine assessment indicated that *Sceletium* possesses mild anti-inflammatory properties but does not hinder the mounting of an adequate immune response to acute immune challenge. These findings indicate that *Sceletium* may be beneficial for the attenuation of cytokine-induced depression, as well as in systemic low-grade inflammation.

**Key words: *Sceletium tortuosum*, alternative medicine, anti-inflammatory, cytokine, monocyte, LPS**

# Abbreviations

| | |
|---|---|
| 5-HT | 5-Hydroxytryptamine |
| ANOVA | Analysis of Variance |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FBS | Foetal Bovine Serum |
| HBSS | Hank's Balanced Salt Solution |
| IDO | Indoleamine 2,3 dioxygenase |
| IFN-γ | Interferon-Gamma |
| IL-1, IL-6 etc. | Interleukin-1, interleukin-6 Etc. |
| LPS | Lipopolysaccharide |
| MCP-1 | Monocyte Chemotactic Protein-1 |
| PBMC(s) | Peripheral Blood Mononuclear Cell(s) |
| RPMI media | Roswell Park Memorial Institute media |
| SEMs | Standard Error of the Mean(s) |
| TNF-α | Tumour Necrosis Factor-Alpha |
| Tri | Trimesemine™ |
| XTT | 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide |

# Introduction

According to the latest World Health Organisation statistics, an estimated 4% of the global population is afflicted with depression. With this number ever increasing, the search for new and effective ways to address the symptoms and modulate both central and peripheral maladaptations to stress-related conditions, remains one of the most important focus areas in research. Although medications are currently available for the management of depressive conditions, less than half (and in many developing countries, less than 10%) of the afflicted population receive such treatments.

In South Africa, as in many developing countries, general practitioners are largely outnumbered by traditional healers. Therefore, it is not surprising that a tendency exists for patients to turn to traditional plant and herbal remedies for the treatment of ailments and disorders (Morris, 2001). Thus, the effectiveness of natural medicines with potential anti-stress capacity has been the focus of several research groups for some time, and one candidate which has shown great potential in this setting is *Sceletium tortuosum*.

Traditionally, *Sceletium tortuosum,* among other *Sceletium* species, was used by the Khoisan people of Southern Africa for the relief of toothache and stomach pain (Harvey *et al*., 2011; Loria *et al*., 2014; Murbach *et al*., 2014; Patnala and Kanfer, 2013), and the commercial availability of this herbal supplement has increased considerably as a result of anecdotal claims of its mood-elevating and anxiolytic properties (Shikanga *et al.*, 2012). However, this commercial expansion is associated with increasing concerns relating to the quality of, and consistency across available *Sceletium* products.

It is important to note that the alkaloidal composition of a supplement is a key determinant of both its beneficial and adverse effects (Nell *et al.*, 2013). This being said, the alkaloidal composition of raw *Sceletium* is complex, and further complicated by post-harvesting interventions which have shown to alter plant composition across the majority of herbal supplements (Smith, 2011). *Sceletium tortuosum* is widely available as an herbal extract for daily supplementation, but little information is available regarding the phytochemical contents, which is necessary for quality control.

In terms of its effectiveness in the context of stress, *Sceletium* extract has been shown to have both effectiveness as monoamine releasing agent and selective serotonin reuptake inhibitor (Coetzee *et al*., 2016), and to limit glucocorticoid production via inhibition of specific adrenal p450 enzymes (Swart and Smith, 2016). In addition, rats subjected to experimental restraint stress after being supplemented with crude *Sceletium tortuosum* extract*,* did exhibit less anxiety behaviour. However, they also presented with increased circulating levels of the pro-inflammatory cytokine, interleukin-1-beta (IL-1β) (Smith, 2011). Although the unrefined nature of the product used may have been responsible for this result, this potentially undesired effect of *Sceletium* supplementation may have far-reaching implications for consumers.

According to the Cytokine Hypothesis of Depression, a significant link exists between cytokines and the mediation of depression (Anisman and Merali, 2002; Maes *et al*., 1995; Schiepers *et al*., 2005). It has furthermore been suggested that cytokine inhibitors, aside from their anti-inflammatory effects, may be capable of offsetting depressive symptoms that accompany chronic inflammation (Dantzer, 2004; Yirmiya, 1996). Therefore, the effect of *Sceletium* supplementation on immune cell functionality is an important area of study, as the efficacy of potential treatments for depression may rely on their ability to down-regulate pro-inflammatory cytokine production.

In the current study, it was hypothesised that the anti-depressive and anxiolytic properties claimed for *Sceletium* are exerted via immunomodulation - specifically anti-inflammatory effects. Our aims were therefore

firstly to determine the effects of a commercially available high-mesembrine *Sceletium* extract, Trimesemine™, on primary human monocyte viability, both basally and in the presence of an acute pro-inflammatory stimulus (*Escherichia coli* lipopolysaccharide, LPS), the latter to simulate severe acute inflammatory challenge. The second aim of this study was to investigate the functional capacity of these immune cells, following treatment with Trimesemine™.

# Materials and Methods

## Ethical considerations

Human primary isolated monocytes were employed in this study. Ethical clearance for blood collection was obtained from Stellenbosch University Subcommittee C Human Research Ethics Committee (Study reference X15/05/013). Monocytes were isolated from peripheral blood buffy coats obtained from healthy donors between the ages of 18 and 25 years old, which were provided by Western Province Blood Transfusion Services (South Africa).

## Cell culture

### Preparation of intervention media

A lyophilised extract (Trimesemine™ (Tri)), prepared from a proprietary hybrid (DV17) of *S. tortuosum* (L.) N.E. Br. and *S. expansum* (L.) L. Bolus (family Aizoaceae) using a proprietary method, was obtained from Botanical Resource Holdings Pty (Ltd) affiliate Verve Dynamics (Somerset West, South Africa) (Lot #BTRMA:001/024, manufacturing reference# DV SCIET:E 028/024 (24123) (refer to Swart and Smith, 2016 for the certificate of analysis and quality control data).

We have previously shown a 0.01mg/ml solution to be most beneficial in an *in vitro* setting (Coetzee *et al*., 2016), thus a 0.01mg/ml Trimesemine™ solution was prepared in serum-free Roswell Park Memorial Institute (RPMI) media. The mixture was vortexed for two minutes and filtered through a 0.22µm syringe filter. As representative of a supra-physiological dose, a 1mg/ml solution was also included. For the inflammatory challenge, a 1mg/ml LPS (Sigma Aldrich, L4391) stock solution was made in Hank's Balanced Salt Solution (HBSS), as per manufacturer's instructions.

### Cell propagation

Human peripheral blood mononuclear cells (PBMCs) were separated from the buffy coat using a Histopaque (Sigma Aldrich, 10771) density gradient. Following centrifugation, the PBMCs were collected, and centrifuged over PercollPLUS (Sigma Aldrich, E0414) density gradients. The resulting monocyte-rich layers were then collected and re-suspended in complete RPMI (RPMI 1640 media containing 10% Foetal Bovine Serum (FBS),

1% Penicillin/streptomycin, 1% GlutaMAX (Gibco® by Life Technologies™, 35050-038) and β-mercaptoethanol (Sigma Aldrich, M3148)) (Menck *et al*., 2014).

The purified monocytes were seeded into a 48-well culture plate at a density of 1 x $10^5$ cells/well in complete RPMI. Cells were incubated at 37°C, 5% $CO_2$ and the medium was refreshed every two days until 90% confluence was reached. It was observed that the cultured primary monocytes went through cycles of adherence and non-adherence to the culture plate surface, and thus media refreshment involved aspiration of the supernatant and centrifugation (400 x g, 10 minutes, without brake, room temperature), following which the supernatant was discarded, and the cell pellets re-suspended in fresh complete RPMI and returned to their respective wells.

## Trimesemine™ treatment intervention

At desired confluence levels, supernatant was aspirated and placed in microfuge vials, and the wells washed once with DPBS. The DPBS was aspirated and added to the vials containing the media from each well, to avoid loss of non-adherent monocytes through the washing process. The microfuge vials were centrifuged as previously described, the supernatant removed, and the cell pellets re-suspended in either 0.01mg/ml or 1mg/ml Trimesemine™-containing media, or serum-free media (control groups), and returned to the respective wells.

After 30 minutes, the following were added to each well: (i) LPS stock solution to LPS-control and LPS-stimulated wells to achieve a final LPS concentration of 50ng/ml (Ross *et al*., 2013), and (ii) LPS vehicle (HBSS) to the unstimulated wells. The cells were then incubated for a further 23.5 hours under standard tissue culture conditions. All experiments were performed at least three times, in duplicate.

## Viability testing

The XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) assay is a commonly used test method to indirectly measure cell viability, through assessment of mitochondrial viability (Wang *et al*., 2011). Following the 23.5-hour incubation period, the supernatant was removed from each well and centrifuged. The resulting supernatant was aliquoted and stored at −80°C for subsequent batch analysis, while the remaining cell pellet was re-suspended in XTT solution (1mg/ml) and returned to their respective wells for incubation (4 hours, 37°C).

Following incubation, optical densities were determined at 490nm, using a Universal Microplate Reader (EL800, Bio-Tek Instruments, Inc.) and analysed using *KCjunior for Windows Data Reduction Software (v1.41.3).*

## Cytokine measurement

Cell-free culture supernatant was analysed using a commercial Magnetic Bead Panel assay (custom-designed 6-plex Milliplex MAP Human Soluble Cytokine Receptor Panel, Merck Millipore), as well as an ELISA Kit (BioLegend, 438807), for concentrations of IL-1β, IL-4, IL-6, IL-10, tumour necrosis factor-alpha (TNF-α) and monocyte chemotactic protein-1 (MCP-1), respectively. Cytokine responses were expressed as absolute concentrations in cell culture supernatant.

Quantification of cytokine concentrations was performed based on a standard curve, derived from linear dilution of the manufacturer-supplied cytokine standards. The lower detection limit was 0.8pg/ml for IL-1β, 4.5pg/ml for IL-4, 0.9pg/ml for IL-6, 1.1pg/ml for IL-10, 0.7pg/ml for TNF-α and 1.6pg/ml for MCP-1.

To understand cytokine secretion in the context of relative viability, an index was calculated using the formula below, to correct the level of cytokines produced for the percentage of viable mitochondria:

*Corrected cytokine secretion index* $= \log_{10}(\textit{cytokine concentration/mitochondrial viability (\% control)} \times 100)$

## Statistical Analysis

Results are presented as averages and standard error of the means (SEMs). Effects of Trimesemine™ and LPS stimulation were statistically analysed by assessing normality of data distribution, followed by a non-parametric 2-way ANOVA and LSD *post hoc* tests. If Levene's test for homogeneity of variances rejected the null hypothesis, the Games-Howell test was used as a *post hoc* test instead (Statistica v.12, StatSoft). Differences were considered significant at $p<0.05$.

# Results

The major findings of this study were that Trimesemine™ supplementation significantly increased monocyte viability in the setting of acute immune challenge. In addition, it was observed that Trimesemine™ exerted anti-inflammatory effects at basal level, through up-regulation of monocyte IL-10 secretion but did not interfere with the cells' ability to mount an adequate inflammatory response following LPS exposure.

At both concentrations, Trimesemine™ treatment was not associated with cytotoxic effects on monocytes, as assessed by XTT (Figure 1). In the absence of LPS, a significant increase in mitochondrial viability was observed in the low dose Trimesemine™ treated condition only, indicating a clear dose-response effect of Trimesemine™ supplementation. LPS exerted significant cytotoxic effects on the primary human monocytes at a concentration of 50ng/ml, resulting in a 66% decrease in XTT viability, which was prevented completely by both Trimesemine™ doses.

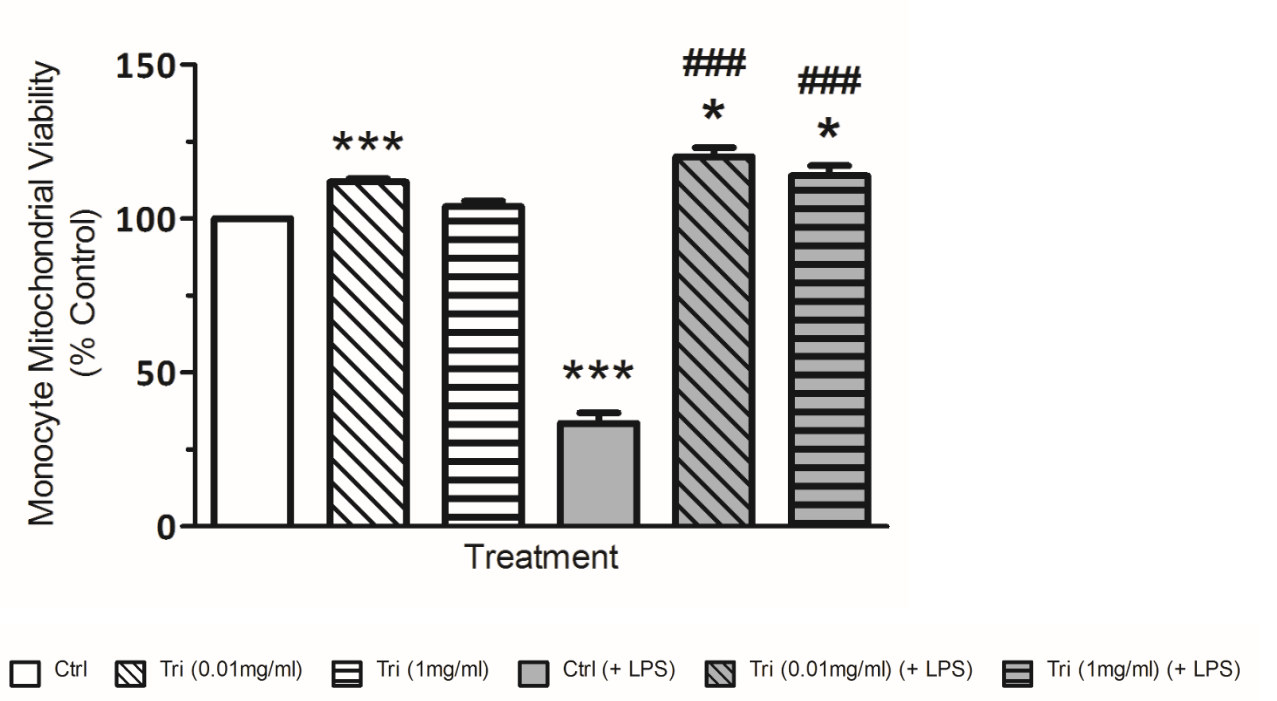


*Figure 1: Dose-dependent primary monocyte mitochondrial viability after exposure to Trimesemine™ with/without LPS for 24 hours. * = p<0.05 (compared to Ctrl); *** = p<0.0001 (compared to Ctrl); ### = p<0.000001 (compared to Ctrl (+ LPS))*

In terms of pro-inflammatory cytokine secretion, monocytes produced detectable absolute levels of IL-1β, IL-6 and TNF-α under basal conditions - a response significantly upregulated for all three cytokines in response to LPS stimulation (Figure 2A-C). Basal secretion levels for MCP-1 was relatively higher than for the other cytokines – which is consistent with the literature (Muenster *et al*., 2015; Ross *et al*., 2013; Weldon *et al*., 2007) – with a dampening effect evident for the high dose Trimesemine™ treatment. Similar to the other cytokines, a significant elevation was seen in response to LPS treatment (Figure 2D).

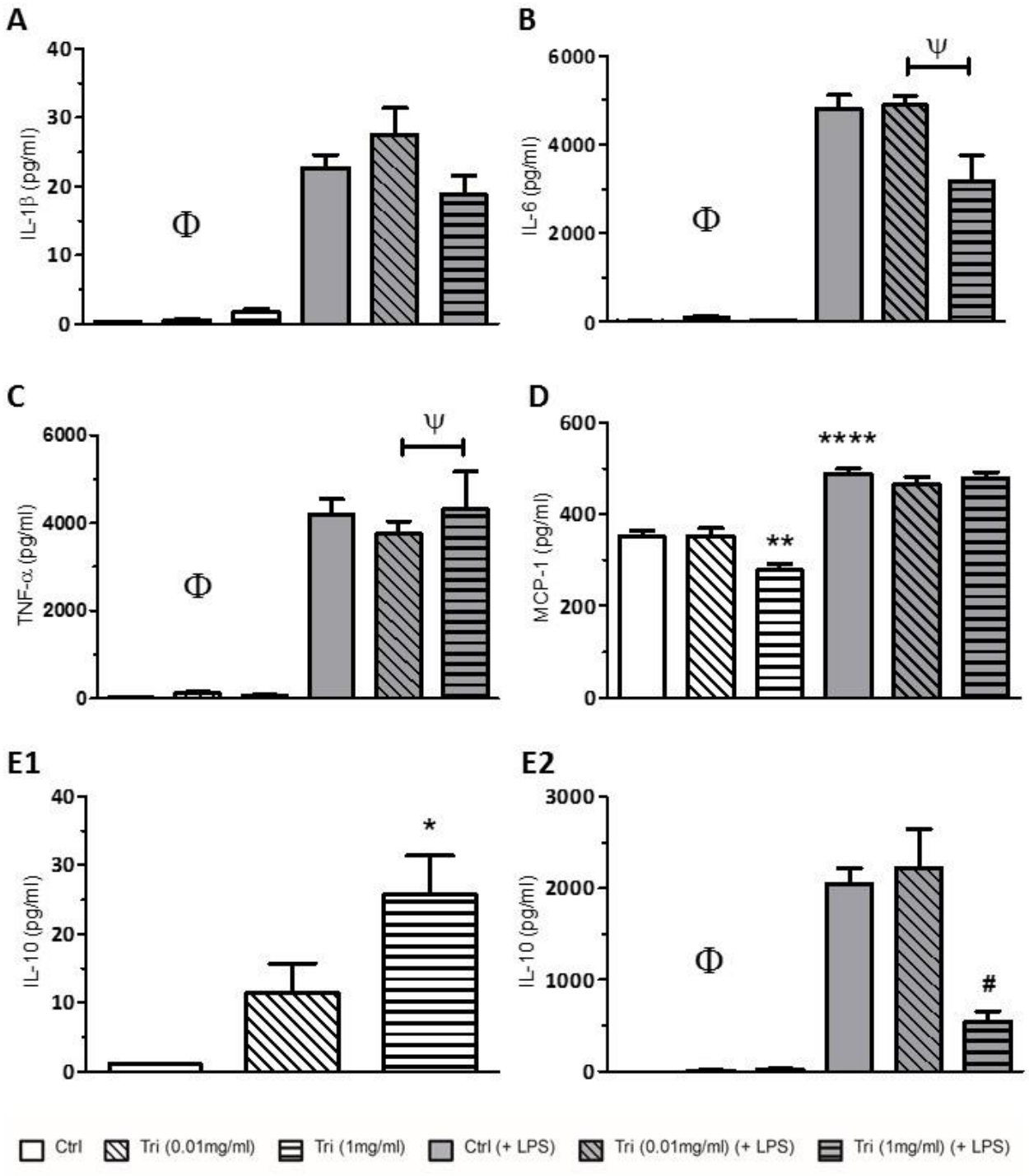


*Figure 2: Effect of Trimesemine™ treatment with/without LPS stimulation on pro-inflammatory (A, B, C and D) and anti-inflammatory (basal only on different axes for clarity, E1 and all conditions, E2) cytokine production by primary human monocytes, as compared to the control. Φ = Main effect of LPS treatment, p<0.01; * = p<0.05 (compared to Ctrl); ** = p<0.005 (compared to Ctrl); **** = p<0.000001 (compared to Ctrl); # = p<0.001 (compared to Ctrl (+ LPS)); ψ = p<0.05*

In comparison to the LPS-stimulated condition, and when considering that the average basal levels for cytokines are very close to the lower detection limits for these assays, monocyte cytokine secretion in response to Trimesemine™ does not give any indication that this extract exerts pro-inflammatory effects under

basal conditions. This interpretation is further supported by the corrected cytokine indices for the pro-inflammatory cytokines (Figure 3A-E; please note that the Y-axes have a log scale).

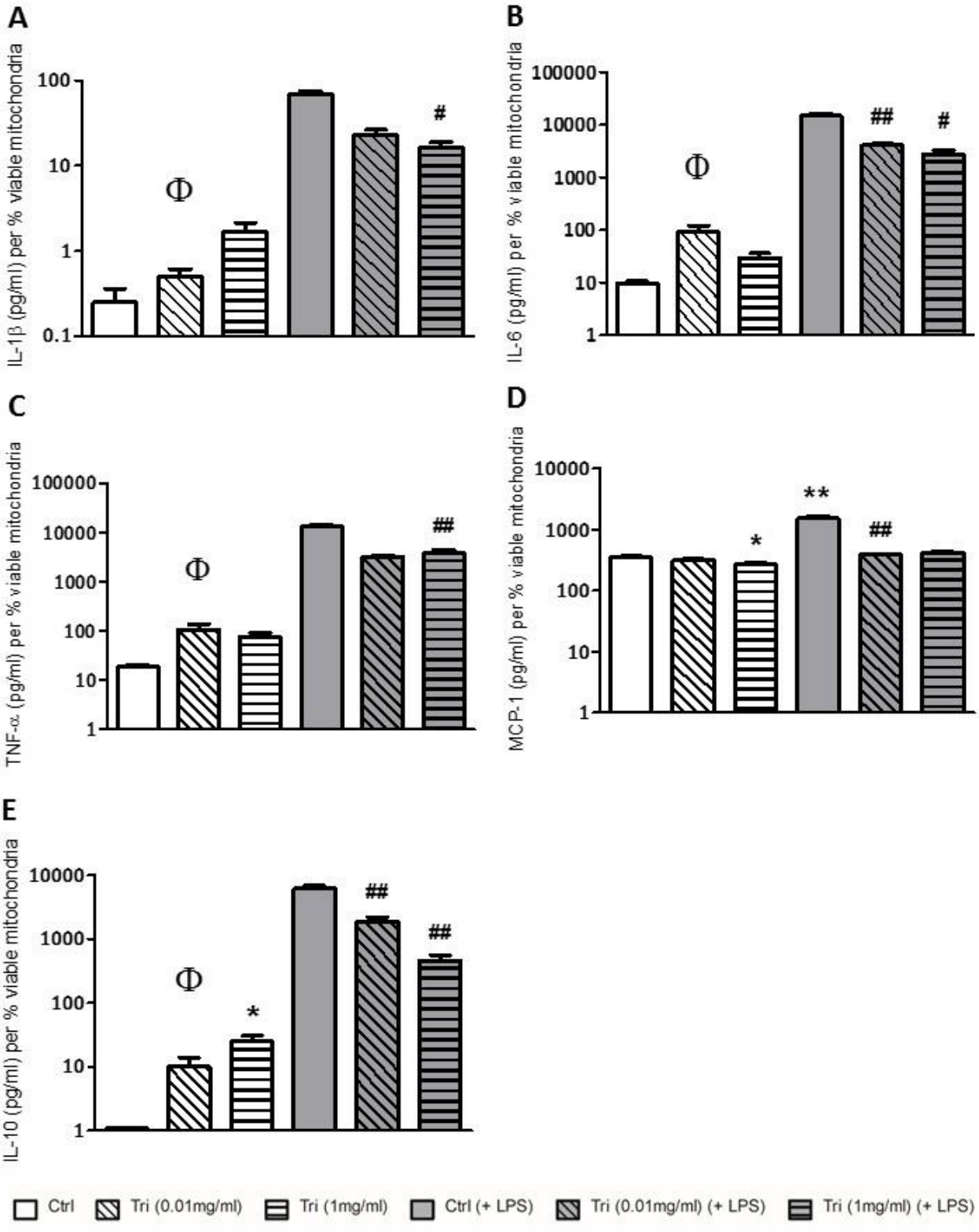


*Figure 3: The monocyte cytokine-viability index per treatment group on a logarithmic scale (base-10). Φ = Main effect of LPS treatment, p<0.01; * = p<0.05 (compared to Ctrl); ** = p<0.005 (compared to Ctrl)); # = p<0.001 (compared to Ctrl (+ LPS)); ## = p<0.005 (compared to Ctrl (+ LPS))*

Under LPS-challenged conditions, neither dose of Trimesemine™ seemed to inhibit absolute pro-inflammatory cytokine concentrations, except for a lower IL-6 response after high dose treatment. However, when correcting cytokine levels according to relative mitochondrial viability, a consistent dose effect emerges, with Trimesemine™ treatment lowering the pro-inflammatory response to LPS challenge (Figure 3A-D). However, although dampened, the response mounted was still significant relative to basal conditions.

Turning to the anti-inflammatory cytokines, IL-4 secretion was below the lowest detectable limit across all treatment groups (data not shown). In contrast, basal IL-10 (Figure 3E1) secretion was significantly increased by Trimesemine™ supplementation in a dose-dependent manner, with statistical significance reached after treatment with the higher dose. In contrast to basal levels, under LPS treated conditions, Trimesemine™ treatment was associated with lower IL-10 secretion, most probably as a result of the lower pro-inflammatory response to LPS in the treated groups.

## Discussion

The current study presents a novel approach to the elucidation of *Sceletium's* potential immunomodulatory properties, firstly through the simultaneous investigation of pro- and anti-inflammatory cytokine production, which to our knowledge has not been performed in other immune cell dose-response ethnopharmacological studies in the context of *Sceletium*. This was motivated by the fact that individual cytokines have overlapping regulatory actions which may be synergistic, additive or antagonistic (Balkwill and Burke, 1989; Cavaillon, 1994), emphasising the importance of studying a broad range of cytokines. Secondly, this study elucidated the effects of a mesembrine-rich commercial *Sceletium* product on leukocyte basal and acute stimulus-induced inflammatory cytokine production, which to our knowledge has not been previously reported in the literature.

The rationale for the use of monocytes as a model was that they are a major source of cytokines, and are characterised by maximal cytokine synthesis within hours of activation (Beutler, 2004; Cavaillon, 1994). It has been observed that these cells are capable of production and release of an impressive panel of cytokines, including, but not limited to, IL-1α, IL-β, IL-6, IL-8, IL-12, TNF-α, interferon-gamma (IFN-γ) and MCP-1 (Cavaillon, 1994).

The fact that monocyte mitochondrial viability decreased significantly after LPS challenge validates the sensitivity of the model to reflect changes in cell viability. Current data indicates that Trimesemine™ did not exert cytotoxic effects at either dosage under basal conditions.

Furthermore, and most importantly contributing to the literature evidence for another major physiological effect of *Sceletium*, Trimesemine™ treatment conferred cytoprotective effects onto immune cells in the presence of acute endotoxin stimulation, thus maintaining XTT viability at control levels. The significance of this result is considerable. Firstly, LPS is known to activate immune cells such as monocytes primarily by binding to Toll-like receptor 4, resulting in activation of intracellular kinases and transcription factors, such as

mitogen-activated protein (MAP) kinases and NF-κB, respectively (Lu *et al*., 2010). The resultant immune response is characterised by release of pro-inflammatory cytokines, nitric oxide and eicosanoids, all of which upregulates the immune response required for removal of the pathogen. However, it is known that normal inflammatory processes results in significant secondary damage to host cells themselves (Smith et al., 2008), as indeed evidenced by the significant drop in XTT viability in the LPS-control cells.

Current data thus suggests that *Sceletium* may protect host immune cells from this secondary damage, thereby effectively enabling the immune system to more efficiently rid the host of the pathogen, with less severe clinical symptoms. Secondly, the fact that the immune cells are protected from the nitric oxide (a free radical) that they secrete in response to LPS, also have implications in the context of sterile inflammation. It is known that many chronic diseases are characterised by increased levels of oxidative stress and low-grade inflammation. Thus, the potential for *Sceletium* to also have antioxidant capacity in this context, in combination with its illustrated anti-stress and anti-anxiety effects (Smith, 2011; Terburg et al., 2013), suggests that this plant medicine may be the magic bullet with which to counter the effects of many modern lifestyle-associated diseases. In further support of this notion, the striking cytoprotective effect of *Sceletium* was associated with significant desired modulatory effects in the context of inflammatory signalling.

The fact that Trimesemine™ up-regulated basal IL-10 secretion, may indicate that this *Sceletium* extract possesses mild anti-inflammatory potential. Importantly, despite this effect, the monocytes could mount an adequate response to LPS stimulation in the presence of Trimesemine™, with a significant up-regulation of IL-10 secretion in response to increased IL-6 production induced by LPS. This result is positive, as it provides evidence to suggest that although a mild modulation by Trimesemine™ under basal conditions may have significance in terms of longer term clinical outcome, its consumption will not hinder the immune response to an acute, pathogenic challenge.

Interestingly, when considering absolute cytokine levels, high-dose Trimesemine™ appeared to result in a smaller IL-6 response to LPS, which indirectly resulted also in decreased monocyte IL-10 secretion. However, when corrected for mitochondrial viability, this dose-effect was not maintained. This suggests that even small (statistically insignificant) differences in mitochondrial viability may affect the cell's functional capacity to produce and/or secrete pro-inflammatory cytokines significantly. The more limited inflammatory cytokine response to LPS in the presence of higher dose Trimesemine™ may be interpreted in two ways. Firstly, the higher dose may limit the undesired symptomatic effects of the inflammatory hyper-response to LPS, which is a favourable clinical outcome. Secondly however, it is possible that this reduced response may be the result of detrimental immunosuppression. However, this seems unlikely, since the cytokine response was still significantly increased in response to LPS when compared to control. Rather, since the high dose employed in the current study represents a supraphysiological dose at least 100-fold higher than suggested daily consumption, this data suggest low risk of overdose, at least in terms of immune capacity and resistance to pathogenic infection.

In terms of the low levels of IL-4 in the current study, a relatively short serum half-life of approximately 20 minutes was reported for IL-4 following intravenous administration (Conlon *et al*., 1990). This could account for the undetectable levels of IL-4 across all treatment groups in the current study, where cytokine assessments were carried out only after a 24-hour intervention. This finding necessitates a need for more time points during the treatment period, to more comprehensively elucidate the secretion peaks for each relevant cytokine. This also indicates that IL-10 serves as a more viable marker for anti-inflammatory profile, as it provides more conclusive results when assessment of fewer time points apply.

To place current results in context with other physiological effects reported for *Sceletium*, exposure to psychological stress has been implicated in defects in production of pro-inflammatory cytokines (Anisman and Merali, 2002; Maes *et al*., 1995; Schiepers *et al*., 2005). It is hypothesised that immune activation may be related to depression-related neurotransmission defects, as pro-inflammatory cytokines themselves have been implicated in alterations in norepinephrine and serotonin/5-hydroxytryptamine (5-HT) turnover in brain regions known to be involved in depression, including the amygdala, hypothalamus, hippocampus and prefrontal cortex (Anderson and Maes, 2015; Dantzer, 2004; Godbout et al., 2005; Poon et al., 2015).

Cytokines are responsible for the lowered activity of presynaptic 5-HT neurons, also due to their interference with serotonin metabolism. Reduced serotonin availability is a result of one or more of the following: (i) the serotonin precursor, tryptophan, is preferentially used for leukocyte activation and synthesis of acute phase proteins, instead of for 5-HT synthesis; (ii) indoleamine 2,3 dioxygenase (IDO), an important enzyme in tryptophan catabolism, is induced by cytokines such as IFN-γ and IL-6; and (iii) low levels of serum albumin, which is essential for tryptophan transport to the blood-brain barrier (Godbout et al., 2005; Maes et al., 1997). In conjunction with reduced availability of 5-HT, upregulation of post-synaptic cleft serotonin receptors has also been observed in the setting of chronic inflammation (Maes et al., 2011).

In addition to their effects on neurotransmitters, cytokines may also influence depression development through their stimulatory actions on the hypothalamic-pituitary-adrenal axis and downregulation of glucocorticoid receptors, resulting in hypercortisolaemia (Maes *et al.*, 1997).

In this context, one can appreciate the beneficial potential of a supplement a combination of selective serotonin-reuptake inhibition, monoamine releasing, anti-oxidant and mild anti-inflammatory properties. *Sceletium tortuosum* is already known to be an effective mood-elevating natural product (Harvey et al., 2011; Loria et al., 2014; Smith, 2011), but this current study has revealed a second positive effect of *Sceletium* supplementation – namely that it may directly and beneficially target immune cells in the peripheral compartment. These findings indicate that *Sceletium* may act as a double-edged sword in attenuation of cytokine-induced depression, as well as in psychologically-induced systemic low-grade inflammation. Importantly, the current study also provides evidence that the beneficial physiological effects of this plant medicine extends beyond its psychoactive effects.

The current study design did not allow for the elucidation of specific plant constituents responsible for these effects. This warrants further investigation in purpose-designed experiments and *in vivo* supplementation studies. In our opinion, in addition to the different alkaloids generally accepted to be the active constituents, anti-oxidant profiling should also be performed, to further elucidate physiological mechanisms of action of this promising plant medicine.

## Conclusion

In conclusion, data presented here suggests that a high-mesembrine *Sceletium* extract, Trimesemine™, beneficially modulates the basal inflammatory cytokine profile without hindering an acute response to pathogenic challenge. Furthermore, data suggests that the extract may affect anti-oxidant activity conferring significant cytoprotection from endotoxin-associated oxidative stress damage. Given the recent reports illustrating increased LPS levels not only in pathogenic infection, but also in chronic diseases such as Alzheimer's disease (Zhao et al., 2017), arthritis (Azzouz and Silverman, 2017) and depression (Zhou et al., 2017), data suggests a potential broader role for Trimesemine™ in the sphere of preventative supplementation.

## Acknowledgements

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## Declaration of Interest

All authors declare no conflict of interest.

## References

See Chapter 6.

# Appendix B – Formulation method for Milliplex kits with diluted beads

## B1 Preparation of reagents for immunoassay:

1. Preparation of antibody-immobilized beads:
   a) Sonicate each antibody-bead vial for 30 seconds and vortex for one minute
   b) Add 45µl from each antibody-bead vial to the Mixing Bottle and bring final volume to 2800µl with Bead Diluent. Vortex the mixture well (Note: this step must be repeated per plate)
2. Preparation of Detection antibodies:
   a) Add 1600µl of detection antibodies (stock) to 1200µl of Assay Buffer, to achieve a total volume of 2800µl (Note: this step must be repeated per plate)
3. Preparation of Quality Controls:
   a) Reconstitute Quality Control 1 and Quality Control 2 with 250µl deionized/distilled water (Note: this step does not need to be repeated per plate)
   b) Invert the vials several times to mix, and vortex
   c) Allow vials to sit for 5-10 minutes, then transfer to appropriately-labelled polypropylene microfuge vials
4. Preparation of Wash Buffer:
   a) Bring the 10X Wash Buffer to room temperature and mix to bring all the salts into solution
   b) Dilute 30ml of 10X Wash Buffer with 270ml deionized/distilled water
5. Preparation of Human Cytokine Standard
   a) Reconstitute the Human Cytokine Standard with 250µl deionized water to give a 10000pg/ml concentration of standard for all analytes
   b) Invert the vial several times to mix, and vortex for 10 seconds
   c) Allow vial to sit for 10 minutes, then transfer to appropriately-labelled polypropylene microfuge vial
6. Preparation of Working standards:
   a) Label five polypropylene microfuge vials: 2000pg/ml, 400pg/ml, 80pg/ml, 16pg/ml and 3.2pg/ml
   b) Add 200µl Assay Buffer to each tube
   c) Prepare serial dilutions according to the following table, vortexing in between each transfer:

| | Standard concentration (pg/ml) | Volume of deionised water (µl) | Volume of standard |
|---|---|---|---|
| 1 | 10000 | 250 | - |
| 2 | 2000 | 200 | 50µl of standard 1 |
| 3 | 400 | 200 | 50µl of standard 2 |
| 4 | 80 | 200 | 50µl of standard 3 |
| 5 | 16 | 200 | 50µl of standard 4 |
| 6 | 3.2 | 200 | 50µl of standard 5 |

## B2 Immunoassay procedure (per plate):

1. Allow all reagents to warm to room temperature (20-25°C) before use in the assay
2. Add 200µl Wash Buffer into each well of the plate
3. Seal and mix on plate shaker for 10 minutes at room temperature
4. Decant wash buffer and remove residual amount from all wells by inverting the plate and tapping it smartly several times, onto absorbent towels
5. Add 25µl of each Standard or Quality Control into the appropriate wells, and Assay Buffer to the 0pg/ml standard (background) wells
6. Add 25µl of Assay Buffer to the sample wells
7. Add 25µl appropriate control culture medium or serum matrix to the background, standards and quality control wells
8. Add 25µl of cell culture supernatant to the appropriate wells (when NAP2 is measured in plasma, samples should be diluted 1:100 in Assay Buffer. Accordingly, serum matrix diluted 1:100 should also be used for the standards and quality control wells)
9. Vortex Mixing Bottle and add 25µl of prepared beads to each well
10. Seal plate, wrap plate in foil and incubate with agitation on plate shaker for two hours at room temperature
11. Wash plate two times using Bioplex Pro Wash station
12. Add 25µl of the prepared Detection Antibodies into each well
13. Seal, cover with foil and incubate with agitation on plate shaker for one hour at room temperature
14. Add 25µl Streptavidinn-Phycoerythrin to each well
15. Seal, cover with foil and incubate with agitation on plate shaker for 30 minutes at room temperature
16. Wash plate two times using Bioplex Pro Wash station
17. Add 75µl Sheath Fluid to all wells
18. Re-suspend beads on plate shaker for five minutes
19. Run plate on the Bio-Plex 200 or MAGPIX instrument

## B3 Equipment settings:

- Bio-Plex 200 (with *Bio-Plex Manager v6.1* software) or Bio-plex MAGPIX (with *Bio-Plex MP* software)
- Beads: 50, per region
- Sample size: 50µl
- Gate settings: Default
- photomultiplier tube (PMT) settings: Default (Low RP1)
- Time out: 60 seconds

# Appendix C – Information Leaflet and Consent Form

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## STELLENBOSCH UNIVERSITY
## CONSENT TO PARTICIPATE IN RESEARCH

**Investigation of *Sceletium tortuosum*'s SSRI/MRA capacity in a platelet model of the central serotonergic system**

You are asked to participate in a research study conducted by Amber Bennett (MSc Physiological Sciences candidate) and Professor Carine Smith (Supervisor), from the Department of Physiological Sciences at Stellenbosch University. The results of this study will contribute towards Amber Bennett's MSc thesis. You were selected as a possible participant in this study because you are a postgraduate student between the ages of 21 and 35, are apparently healthy, do not smoke, and are able to donate blood for our research.

### 1. PURPOSE OF THE STUDY

The aim of this study is to determine the effects of *Sceletium tortuosum* treatment on serotonin storage in, and release by, human platelets. *Sceletium tortuosum* has one of the oldest histories of use of any South African medicinal plant, with the earliest written records of use dating back to 1662. In the Western world, *Sceletium tortuosum* has been used in numerous settings, including the management of anxiety and depression, as it anecdotally possesses mood-elevating properties.

This study will provide us with very important information on *Sceletium tortuosum's* anti-inflammatory and anti-oxidant capacity, as well as its effects on neurotransmitter release within the body.

Furthermore, this study may provide us with information regarding *Sceletium tortuosum's* effects on the clotting capacity of platelets. This information would indicate potential alterations in the blood that may be caused by regular *Sceletium tortuosum* supplementation.

### 2. PROCEDURES

If you volunteer to participate in this study, we would ask you to do the following things: First, we require you to fill out two simple questionnaires that tell us a bit more about your lifestyle. One of them will focus on your overall health, how regularly you exercise, and what medications you are currently

taking. The second questionnaire will be used to assess your anxiety levels and your overall anxiety proneness. **Please note: these questionnaires do ask you to disclose information regarding sensitive issues such as depression, cancer, chronic diseases, and family history of disease.**

From the questionnaires, we will be able to decide if you may participate in this study or not. If you meet the criteria, you will then be asked to give us a blood sample (±20ml or 2 tablespoons of blood), which will be drawn by a certified phlebotomist within the Department of Physiological Sciences. This should not take more than 10-15 minutes in total.

Using your blood sample, we will perform a full blood count and a clotting profile, to ensure that your blood is suitable for this experiment. If we find any abnormalities, you will be informed immediately and referred to a doctor at Campus Health for further evaluation.

If your full blood count and clotting profile results are satisfactory, we will then ask you to please come back to the department, where we will take another blood sample (±20ml or 2 tablespoons of blood). This procedure will also not take more than 10-15 minutes in total. We will then isolate the platelets from your blood for further treatment with *Sceletium tortuosum*.

**Please note: Before each blood sample, you <u>may not</u> consume any alcohol and/or drugs, or any medication, supplements or vitamins at least one week prior to the blood draw. We will arrange a date for the blood draws so that you have enough time to avoid these things in advance.**

## 3. POTENTIAL RISKS AND DISCOMFORTS

The normal, minimal risk of bruising and infection associated with blood draws will exist, but since experienced phlebotomists will be taking blood samples, we are confident that we have minimised this risk. In the event of a participant exhibiting abnormal clotting times, participation in the study will be discontinued immediately.

In the unlikely event of some form of injury occurring, a departmental investigation will be done and necessary steps shall be taken, keeping in consideration the participant's best interests. In the case of a medical emergency, you will be referred to Campus Health for a consultation and/or treatment.

## 4. POTENTIAL BENEFITS TO SUBJECTS AND/OR TO SOCIETY

You will not directly benefit by participating in this study. However, your blood samples will be contributing to important research being conducted by the Department of Physiological Sciences at Stellenbosch University.

The results from this research may benefit society by providing information regarding the effects of *Sceletium tortuosum* consumption, particularly for those that tend to consume *Sceletium tortuosum* products on a regular basis.

The information gained from this study may also help us determine which compounds within the *Sceletium tortuosum* plant are most beneficial for human health, in terms of inflammation and neurotransmitter release.

## 5. PAYMENT FOR PARTICIPATION

You will not be paid to participate in this study.

There will be no costs involved for you, if you choose to participate in this study.

## 6. CONFIDENTIALITY

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law.

Confidentiality will be maintained by means of ascribing a unique code to your data, that is known only by Amber Bennett and Prof Smith. Your questionnaires will be stored safely and securely in Prof Smith's office, which will remain locked at any time that she is not present in the office. If the results of this study are published, your identifying details will remain confidential and will not be included in the publication.

In the case of a medical emergency, your information may be provided to the health practitioner that will be involved in your consultation and/or treatment.

## 7. PARTICIPATION AND WITHDRAWAL

You can choose whether to be in this study or not. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind. The investigator may withdraw you from this research if circumstances arise which warrant doing so.

## 8. IDENTIFICATION OF INVESTIGATORS

If you have any questions or concerns about the research, please feel free to contact the Principle Investigator, Amber Bennett, at any time (16429737@sun.ac.za; 082 868 8079). You may also contact Prof Carine Smith during office hours (csmith@sun.ac.za; 021 808 4388).

## 9. RIGHTS OF RESEARCH SUBJECTS

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. If you have questions regarding your rights as a research subject, contact Ms Maléne Fouché (mfouche@sun.ac.za; 021 808 4622) at the Division for Research Development.

## SIGNATURE OF RESEARCH SUBJECT OR LEGAL REPRESENTATIVE

The information above was described to me by Miss Amber Bennett in [Afrikaans/English/Xhosa/other] and I am in command of this language or it was satisfactorily translated to me. I was given the opportunity to ask questions and these questions were answered to my satisfaction.

I hereby consent voluntarily to participate in this study.

I have been given a copy of this form.

_____
Name of Participant

_____
Name of Legal Representative (if applicable)

_____ _____
**Signature of Participant or Legal Representative** **Date**

## SIGNATURE OF INVESTIGATOR

I declare that I explained the information given in this document to _____ [*name of the participant]* and/or [his/her] representative _____ [*name of the representative]*. *[H*e/she] was encouraged and given ample time to ask me any questions. This conversation was conducted in [*Afrikaans/*English/*Xhosa/*Other*] and no translator was used.

_____ _____
**Signature of Investigator** **Date**

# Appendix D – Health and Lifestyle Questionnaire

Participant code #:_____

## HEALTH AND LIFESTYLE QUESTIONNAIRE

These questions are intended to obtain some personal health information from those that are interested in taking part in the study.

We are looking for people that fit specific criteria, so please try to answer as truthfully as possible. If you are unsure of any of the questions, please check with the Investigator before omitting/completing an answer.

All information will remain private and confidential.

| General information | |
|---|---|
| Age | |
| Gender | |
| Weight | |
| Height | |

| Stress and anxiety | |
|---|---|
| How would you rate your current stress level on a scale of 1 to 10? (1 being calmest, 10 being totally stressed out) | 1 2 3 4 5 6 7 8 9 10 |
| How do you manage your stress? (exercise, medication, TV etc.) | |
| Are you currently experiencing depression and/or anxiety? | YES / NO |

| Lifestyle | |
|---|---|
| Do you smoke? If yes, how many cigarettes per day? | YES / NO _____ per day |

| | |
|---|---|
| Do you drink? If yes, how many units per week?<br>(1 unit = 1 glass of wine, 1/2 pint of beer, 1 measure of spirits) | YES / NO _____ units per week |
| How many times a week do you exercise? | |
| For how long do you exercise in one exercise session? | |

| **Family History**<br>Have any of your first degree relatives (mother, father, sisters, brothers) ever been diagnosed with: | |
|---|---|
| Cancer | YES / NO |
| Depression and/or anxiety | YES / NO |
| Heart disease | YES / NO |
| High blood pressure | YES / NO |

| **Health Profile** | |
|---|---|
| Have you had a medical checkup within the last 12 months? | YES / NO |
| Have you had an organ transplant? | YES / NO |
| Do you have any inflammatory diseases/allergies (asthma, hayfever etc.)? | YES / NO |
| Are you currently experiencing any cold- or flu-like symptoms? | YES / NO |

| **Chronic medication**<br>Are you currently taking any of the following on an acute/chronic basis: | |
|---|---|
| Immune-suppressant medication (anti-inflammatories, cortisone etc.) | YES / NO |
| Anti-coagulant medication (aspirin, warfarin etc.) | YES / NO |

| | |
|---|---|
| Anti-depressants or anti-anxiety medication | YES / NO |
| Contraceptive or fertility medication | YES / NO |
| Other prescription/non-prescription medications (Please list) | |

# Appendix E – State-Trait Anxiety Index Questionnaire

## State-Trait Anxiety Inventory for Adults

### Self-Evaluation Questionnaire

STAI Form Y-1 and Form Y-2

**Developed by Charles D. Spielberger**
in collaboration with R.L. Gorsuch, R. Lushene, P.R. Vagg, and G.A. Jacobs



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www.mindgarden.com

## SELF-EVALUATION QUESTIONNAIRE STAI Form Y-1

**Please provide the following information:**

Name_____Date_____S_____

Age_____ Gender (*Circle*) **M** **F** T_____

**DIRECTIONS:**

A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you feel *right* now, that is, *at this moment*. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best.

| | NOT AT ALL | SOMEWHAT | MODERATELY SO | VERY MUCH SO |
|---|---|---|---|---|
| 1. I feel calm | 1 | 2 | 3 | 4 |
| 2. I feel secure | 1 | 2 | 3 | 4 |
| 3. I am tense | 1 | 2 | 3 | 4 |
| 4. I feel strained | 1 | 2 | 3 | 4 |
| 5. I feel at ease | 1 | 2 | 3 | 4 |
| 6. I feel upset | 1 | 2 | 3 | 4 |
| 7. I am presently worrying over possible misfortunes | 1 | 2 | 3 | 4 |
| 8. I feel satisfied | 1 | 2 | 3 | 4 |
| 9. I feel frightened | 1 | 2 | 3 | 4 |
| 10. I feel comfortable | 1 | 2 | 3 | 4 |
| 11. I feel self-confident | 1 | 2 | 3 | 4 |
| 12. I feel nervous | 1 | 2 | 3 | 4 |
| 13. I am jittery | 1 | 2 | 3 | 4 |
| 14. I feel indecisive | 1 | 2 | 3 | 4 |
| 15. I am relaxed | 1 | 2 | 3 | 4 |
| 16. I feel content | 1 | 2 | 3 | 4 |
| 17. I am worried | 1 | 2 | 3 | 4 |
| 18. I feel confused | 1 | 2 | 3 | 4 |
| 19. I feel steady | 1 | 2 | 3 | 4 |
| 20. I feel pleasant | 1 | 2 | 3 | 4 |





STAIP-AD Test Form Y
www.mindgarden.com

## SELF-EVALUATION QUESTIONNAIRE

**STAI Form Y-2**

Name_____ Date_____

### DIRECTIONS

A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you *generally* feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you generally feel.

| | ALMOST NEVER | SOMETIMES | OFTEN | ALMOST ALWAYS |
|---|---|---|---|---|
| 21. I feel pleasant | 1 | 2 | 3 | 4 |
| 22. I feel nervous and restless | 1 | 2 | 3 | 4 |
| 23. I feel satisfied with myself | 1 | 2 | 3 | 4 |
| 24. I wish I could be as happy as others seem to be | 1 | 2 | 3 | 4 |
| 25. I feel like a failure | 1 | 2 | 3 | 4 |
| 26. I feel rested | 1 | 2 | 3 | 4 |
| 27. I am "calm, cool, and collected" | 1 | 2 | 3 | 4 |
| 28. I feel that difficulties are piling up so that I cannot overcome them | 1 | 2 | 3 | 4 |
| 29. I worry too much over something that really doesn't matter | 1 | 2 | 3 | 4 |
| 30. I am happy | 1 | 2 | 3 | 4 |
| 31. I have disturbing thoughts | 1 | 2 | 3 | 4 |
| 32. I lack self-confidence | 1 | 2 | 3 | 4 |
| 33. I feel secure | 1 | 2 | 3 | 4 |
| 34. I make decisions easily | 1 | 2 | 3 | 4 |
| 35. I feel inadequate | 1 | 2 | 3 | 4 |
| 36. I am content | 1 | 2 | 3 | 4 |
| 37. Some unimportant thought runs through my mind and bothers me | 1 | 2 | 3 | 4 |
| 38. I take disappointments so keenly that I can't put them out of my mind | 1 | 2 | 3 | 4 |
| 39. I am a steady person | 1 | 2 | 3 | 4 |
| 40. I get in a state of tension or turmoil as I think over my recent concerns and interests | 1 | 2 | 3 | 4 |





STAIP-AD Test Form Y
www.mindgarden.com

## State-Trait Anxiety Inventory for Adults Scoring Key (Form Y-1, Y-2)

**Developed by Charles D. Spielberger** in collaboration with R.L. Gorsuch, R. Lushene, P.R. Vagg, and G.A. Jacobs

To use this stencil, fold this sheet in half and line up with the appropriate test side, either Form Y-1 or Form Y-2. Simply total the scoring **weights** shown on the stencil for each response category. For example, for question # 1, if the respondent marked 3, then the **weight** would be **2**. Refer to the manual for appropriate normative data.

| Form Y-1 | NOT AT ALL | SOMEWHAT | MODERATELY SO | VERY MUCH SO |
|---|---|---|---|---|
| 1. | 4 | 3 | 2 | 1 |
| 2. | 4 | 3 | 2 | 1 |
| 3. | 1 | 2 | 3 | 4 |
| 4. | 1 | 2 | 3 | 4 |
| 5. | 4 | 3 | 2 | 1 |
| 6. | 1 | 2 | 3 | 4 |
| 7. | 1 | 2 | 3 | 4 |
| 8. | 4 | 3 | 2 | 1 |
| 9. | 1 | 2 | 3 | 4 |
| 10. | 4 | 3 | 2 | 1 |
| 11. | 4 | 3 | 2 | 1 |
| 12. | 1 | 2 | 3 | 4 |
| 13. | 1 | 2 | 3 | 4 |
| 14. | 1 | 2 | 3 | 4 |
| 15. | 4 | 3 | 2 | 1 |
| 16. | 4 | 3 | 2 | 1 |
| 17. | 1 | 2 | 3 | 4 |
| 18. | 1 | 2 | 3 | 4 |
| 19. | 4 | 3 | 2 | 1 |
| 20. | 4 | 3 | 2 | 1 |

| Form Y-2 | ALMOST NEVER | SOMETIMES | OFTEN | ALMOST ALWAYS |
|---|---|---|---|---|
| 21. | 4 | 3 | 2 | 1 |
| 22. | 1 | 2 | 3 | 4 |
| 23. | 4 | 3 | 2 | 1 |
| 24. | 1 | 2 | 3 | 4 |
| 25. | 1 | 2 | 3 | 4 |
| 26. | 4 | 3 | 2 | 1 |
| 27. | 4 | 3 | 2 | 1 |
| 28. | 1 | 2 | 3 | 4 |
| 29. | 1 | 2 | 3 | 4 |
| 30. | 4 | 3 | 2 | 1 |
| 31. | 1 | 2 | 3 | 4 |
| 32. | 1 | 2 | 3 | 4 |
| 33. | 4 | 3 | 2 | 1 |
| 34. | 4 | 3 | 2 | 1 |
| 35. | 1 | 2 | 3 | 4 |
| 36. | 4 | 3 | 2 | 1 |
| 37. | 1 | 2 | 3 | 4 |
| 38. | 1 | 2 | 3 | 4 |
| 39. | 4 | 3 | 2 | 1 |
| 40. | 1 | 2 | 3 | 4 |




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STAIP-AD Scoring Key
www.mindgarden.com

# Appendix F – Platelet isolation optimisation for platelet assay

To optimise platelet isolation for suitable yield as well as low activation, four platelet isolation methods (derived from available literature) were tested. All methods were tested on blood samples from the same individual and were drawn the same day.

Platelets were stained with mouse anti-human CD41a antibodies, as this marker was initially used as a platelet activation marker in coagulation studies. In this context, decreased binding of CD41a antibodies to activated platelets has been previously demonstrated (Gobbi *et al*., 2003). In line with this, CD41a was employed as platelet gating marker in the current study. In addition, it provided information on activation status of platelets.

## F1 Method 1

1. Draw blood via antecubital venous puncture (sodium citrate anti-coagulated)
2. Centrifuge whole blood (200xg, 5 minutes, room temperature, no brake)
3. Remove plasma (yields ±1ml per 4.5ml blood collection tube) and transfer to new sterile 15ml tube
4. Add PBS at 1:1 ratio
5. Perform automated analysis (Celldyne 3700CS, Abbott Diagnostics)
    - Platelet yield: $2.1x10^9$ platelets/L

## F2 Method 2

1. Draw blood via antecubital venous puncture (sodium citrate anti-coagulated)
2. Centrifuge whole blood (200xg, 20 minutes, 20°C, no brake)
3. Remove plasma (yields ±1.5ml per 4.5ml blood collection tube) and transfer to new sterile 15ml tube
4. Perform automated analysis (Celldyne 3700CS, Abbott Diagnostics)
    - Platelet yield: $145x10^9$ platelets/L

## F3 Method 3

1. Draw blood via antecubital venous puncture (sodium citrate anti-coagulated)
2. Centrifuge whole blood (200xg, 20 minutes, room temperature, no brake)
3. Remove plasma (yields ±2ml per 4.5ml blood collection tube) and transfer to new sterile 15ml tube
4. Add PBS at 1:1 ratio
5. Centrifuge (800xg, 20 minutes, room temperature)
6. Resuspend platelet pellet in PBS
7. Perform automated analysis (Celldyne 3700CS, Abbott Diagnostics)
    - Platelet yield: $63.9x10^9$ platelets/L

## F4 Method 4

1. Draw blood via antecubital venous puncture (sodium citrate anti-coagulated)
2. Centrifuge whole blood (400xg, 15 minutes, room temperature, no brake)
3. Remove plasma (yields ±2ml per 4.5ml blood collection tube) and transfer to new sterile 15ml tube
4. Centrifuge (400xg, 15 minutes, room temperature, no brake)
5. Aspirate PPP (upper ±1/3 of plasma)
6. Resuspend platelet pellet in PRP (lower ±2/3 of plasma)
7. Perform automated analysis (Celldyne 3700CS, Abbott Diagnostics)
    - Platelet yield: $46.7x10^9$ platelets/L

Following automated analysis for platelet count (all methods):

1. Add 500µl 1X BD FACS-lyse solution to plasma, incubate for 10 minutes at room temperature
2. Add 1ml PBS into the sample tube
3. Centrifuge (800xg, 20 minutes, room temperature)
4. Remove supernatant and resuspend platelet pellet in 500µl 0.1% saponin, incubate for 10 minutes at room temperature
5. Add 2ml PBS into the sample tube
6. Centrifuge (800xg, 20 minutes, 4°C)
7. Remove supernatant and resuspend platelet pellet in 100µl PBS with 3% FBS
8. Add conjugated antibodies for CD41a
9. Incubate platelets in the dark for 30 minutes at 4°C
10. Perform final wash step with PBS and centrifuge (400xg, 5 minutes, 4°C)
11. Resuspended in 1% paraformaldehyde and assess by flow cytometric analysis

Flow cytometry scatter plots illustrating CD41a+ platelets can be seen in Figure 7.1. Gating parameters were previously determined through fluorescence minus one (FMO) controls and single stain sample analysis (Appendix G).

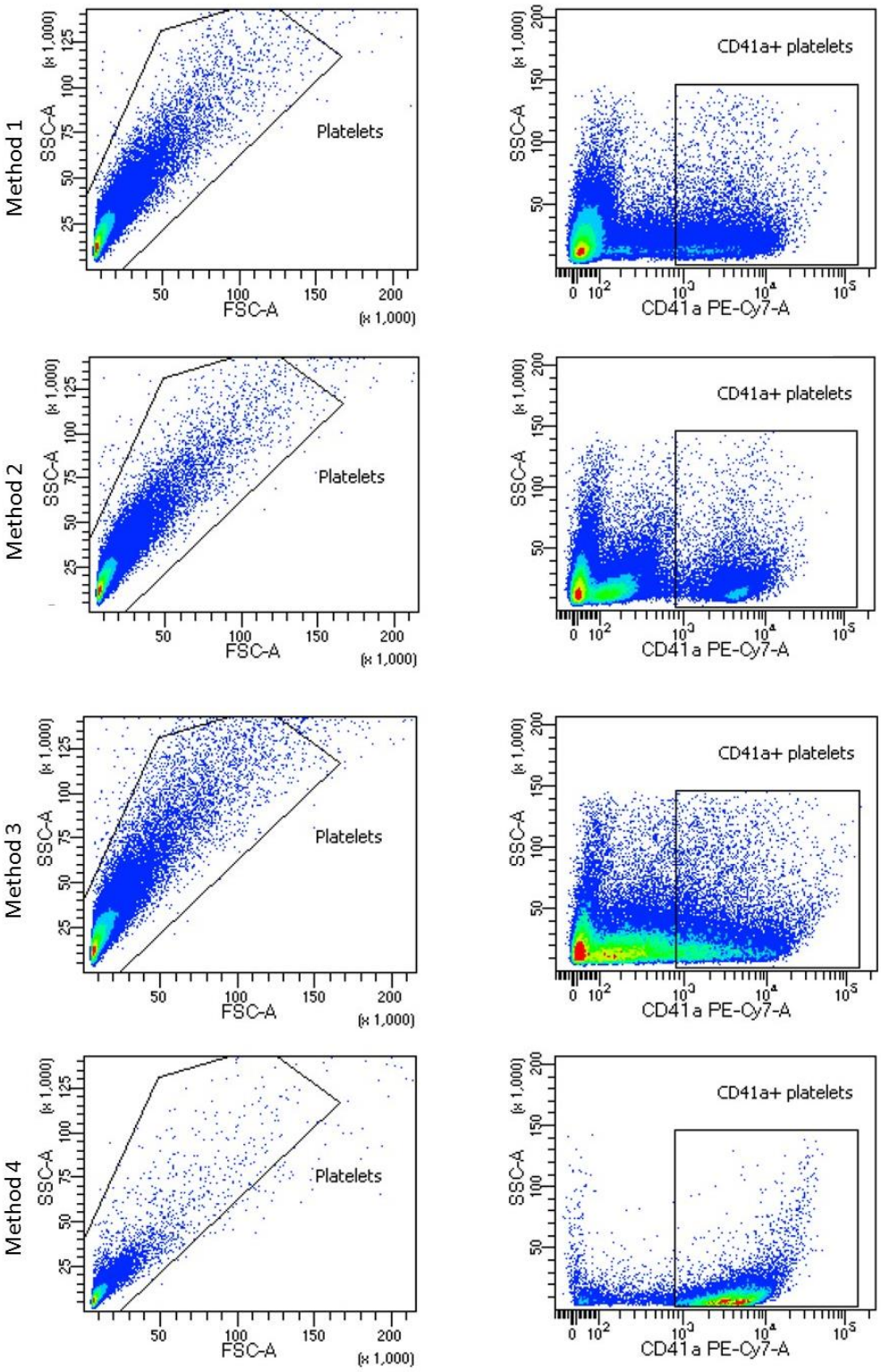


*Figure 7.1 Flow cytometry scatterplots showing platelet gating and CD41a+ platelets*

The results of flow cytometric analysis of activation of the samples acquired through each method, are quantified in Table 7.1.

Table 7.1: Flow cytometry results indicating degree of platelet activation based on CD41a expression

| | CD41a (mean fluorescent intensity) | CD41a+ platelets (% total platelet count) |
|---|---|---|
| Method 1 | 2579 | 15.9 |
| Method 2 | 3833 | 14 |
| Method 3 | 2449 | 26.9 |
| Method 4 | 3859 | 84.5 |

It was concluded that Method 4 was the optimal platelet isolation method to employ, in terms of favourable platelet yield with low platelet activation. This was important for the subsequent platelet assay, where platelet activation in response to treatment exposure would be measured.

# Appendix G – Flow cytometric analysis

## G1 Description of instrument

The BD FACSAria IIu flow cytometer (BD Biosciences, USA) was employed for the analysis of samples, housed in the Central Analytic Facilities Fluorescent Imaging Unit, Stellenbosch University. The instrument is a high-speed benchtop cell sorter, able to acquire up to 70,000 events per second. The flow cytometer is fitted with blue (488-nm), red (633-nm) and violet (405-nm) solid state lasers for multicolour analysis of ≤9 fluorescent markers and two physical parameters, forward scatter (FSC) and side scatter (SSC). It is also equipped with two types of signal detectors; PMTs and a photodiode detector. PMTs detect signals generated by SSC and all fluorescence channels, whereas the photodiode signal generated by FSC.

The BD FACSAria IIu possesses an octagon and two trigon detector arrays. The octagon detector array contains 6 PMTs that detect SSC and up to five fluorescence signals excited by the blue laser. The trigon arrays detect fluorescence signals excited by the red and violet lasers. Each trigon contains two PMTs that detect up to two fluorescence channels. The PMTs within each array convert light into electrical pulses that can be processed by the electronics system and converted into data. Acquisition and data analysis, as well as most BD FACSAria II cytometer functions, are performed on *BD FACSDiva v8.1* software.

Table 7.2 displays the filters (longpass and bandpass), detectors and voltage settings employed in the astrocyte propidium iodide viability assay.

Table 7.2: Instrument parameters for astrocyte propidium iodide viability assay by flow cytometry

| Laser | LP filter | BP filter | Detector | Parameter | Voltage | Amplification |
|---|---|---|---|---|---|---|
| 488nm | n/a | 488/10 | SSC (PD) | SSC-A | 280 | Linear |
| | 502LP | 610/20 | 488-C | BD Horizon™ PE-CF594 | 410 | Log |

The following table displays the filters (longpass and bandpass), detectors and voltage settings employed in the platelet assay:

Table 7.3: Instrument parameters for platelet assay by flow cytometry

| Laser | LP filter | BP filter | Detector | Parameter | Voltage | Amplification |
|---|---|---|---|---|---|---|
| 488nm | n/a | 488/10 | SSC (PD) | SSC-A | 250 | Linear |
| | 502 | 530/30 | 488-F | Alexa Fluor™ 488 | 443 | Log |
| | 595 | 610/20 | 488-C | BD Horizon™ PE-CF594 | 540 | Log |
| | 735 | 780/60 | 488-A | BD Horizon™ PE-Cyanine7 | 580 | Log |
| 633 nm | 735 | 660/20 | 633-B | Alexa Fluor™ 647 | 420 | Log |
| 405nm | 502 | 450/40 | 405-B | Alexa Fluor™ 405 | 449 | Log |

## G2 Antibody information and titration protocol for platelet assay

Characteristics of all antibodies used in the platelet assay are summarised in Table 7.4.

Table 7.4: Platelet assay antibody characteristics

| Marker | Host | Species reactivity | Clonality | Isotype | Fluorochrome |
|---|---|---|---|---|---|
| Serotonin | Rat | Human | Monoclonal | IgG2 | None |
| Anti-rat IgG | Donkey | Rat | Polyclonal | IgG | Alexa Fluor™ 647 |
| SERT | Goat | Human, mouse | Polyclonal | IgG1 | None |
| Anti-goat IgG | Donkey | Goat | Polyclonal | IgG | Alexa Fluor™ 488 |
| VMAT-2 | Mouse | Human | Monoclonal | IgG1 | Alexa Fluor™ 405 |
| CD41a | Mouse | Human | Monoclonal | IgG1 | BD Horizon™ PE-Cyanine7 |
| CD63 | Mouse | Human | Monoclonal | IgG1 | BD Horizon™ PE-CF594 |

To optimise antibody concentrations for platelet flow cytometric analysis, antibody titrations were performed on isolated platelets, activated with calcium ionophore A23187. This ensured that all markers of interest would be expressed. Antibodies were diluted according to the following table:

Table 7.5: Platelet assay antibody titrations

| Marker | Concentration (mg/ml) | Volume (µl) | Recommended (per sample) | Dilution 1 | Dilution 2 | Dilution 3 |
|---|---|---|---|---|---|---|
| Serotonin | 0.5 | 100 | 1:10-1:500 | 1µl | 0.5µl* | 0.25µl |
| Anti-rat IgG | 2 | 500 | 0.5µl | 0.5µl* | - | - |
| SERT | 1 | 200 | 5µl | 2.5µl* | 2µl | 1.5µl |
| Anti-goat IgG | 2 | 500 | 0.5µl | 0.5µl* | - | - |
| VMAT-2 | 0.2 | 100 | 0.25-1µg per $10^6$ cells | 1.25µl* | 1µl | 0.75µl |
| CD41a | 0.0025 | 500 | 5µl | 5µl | 2.5µl* | 1.25µl |
| CD63 | 0.0025 | 500 | 5µl | 5µl* | 2.5µl | 1.25µl |

* Optimal concentrations determined by flow cytometry

## G3 Instrument setup and controls

For compensation controls, single-stained cell controls were prepared for each antibody in the staining panel. The cell controls were then acquired on the BD FACSAria IIu and analysed for the percentage of spill over for each tested antibody. The resulting compensation matrix was then applied to the samples and employed for the analysis of the samples. Table 7.6 illustrates the compensation matrix, expressed as a percentage.

Table 7.6: Compensation matrix for platelet assay by flow cytometry

| | Alexa Fluor™ 488 | BD Horizon™ PE-CF594 | BD Horizon™ PE-Cyanine7 | Alexa Fluor™ 647 | Alexa Fluor™ 405 |
|---|---|---|---|---|---|
| Alexa Fluor™ 488 | 100 | 16.07 | 0.81 | 0.03 | 0.75 |
| BD Horizon™ PE-CF594 | 0.04 | 100 | 12.32 | 0.19 | 0.00 |
| PE BD Horizon™ PE-Cyanine7 | 0.02 | 0.55 | 100 | 0.01 | 0.00 |
| Alexa Fluor™ 647 | 0.00 | 0.00 | 0.63 | 100 | 0.00 |
| Alexa Fluor™ 405 | 0.00 | 0.00 | 0.00 | 0.00 | 100 |

To ensure that the gating strategy employed was able to accurately differentiate true positive and negative populations, FMO controls were performed for the regions of interest.

For the experiment, application settings were used to determine optimal voltage settings for each parameter and as an internal experimental control 8-peak rainbow beads were used to ensure that mean fluorescent intensity (MFI) changes remained constant.

## G4 Sample acquisition and analysis of platelet assay

Before acquisition, the sample tubes and beads were vortexed for 5 seconds to ensure that the cells and beads were well suspended. *BD FACSDiva™* software was used for data acquisition and analysis. A minimum of 40 0000 gated events were collected for each sample tube.

To identify platelets, a FSC-H versus CD41a dot plot was employed and all CD41a+ cells were gated. Within the CD41+ population, doublets were excluded by using a double discrimination gate (FSC-H vs FSC-A), which was performed to exclude clumped cells. All other parameters were determined from the resulting singlet cells (Figure 7.2). For the study, we reported both changes in frequency as well as MFI.

For statistical analysis of the flow cytometric data, data was exported to excel format and incorporated into statistical software.

G5 Representative images of gating for all parameters of interest

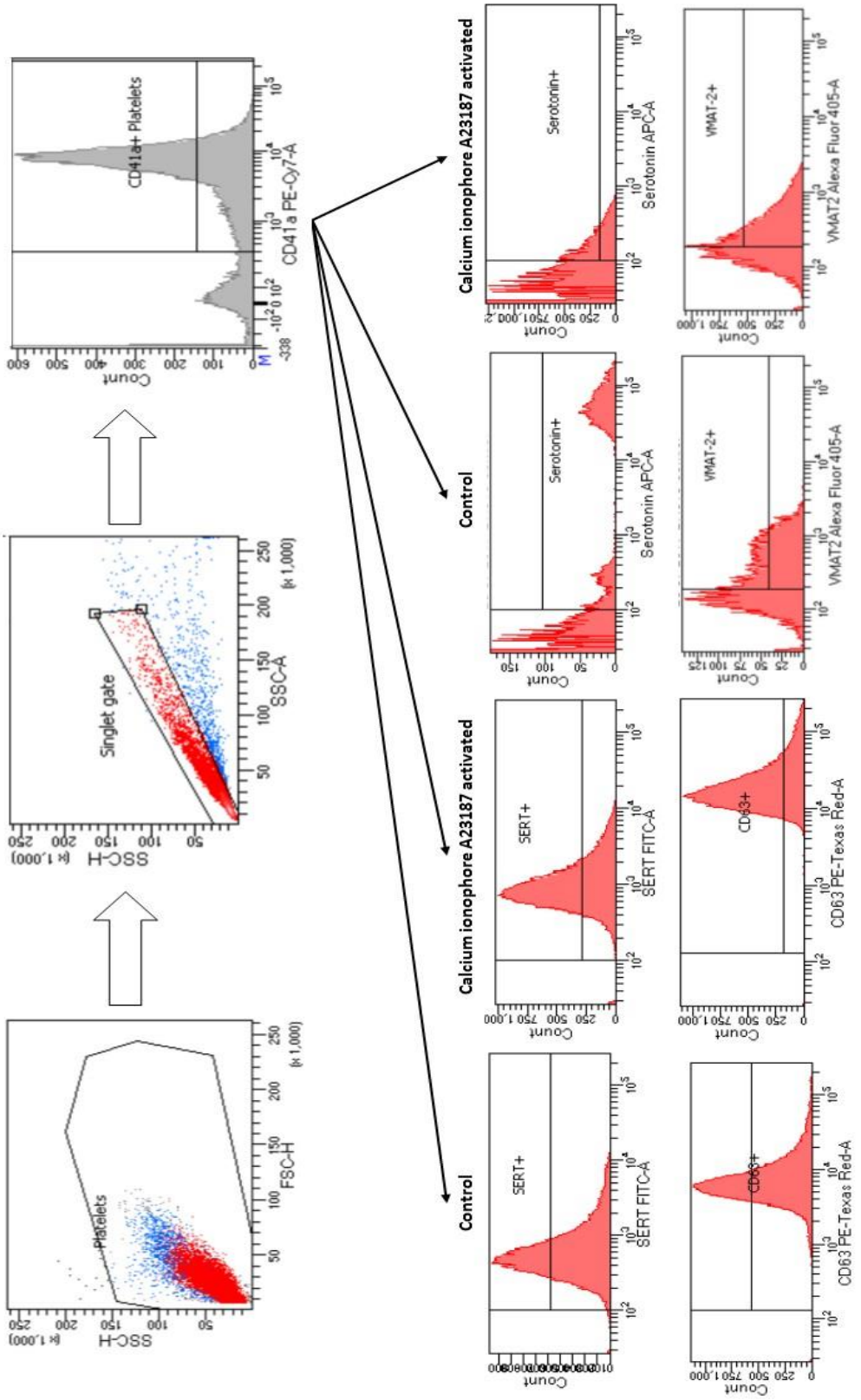


*Figure 7.2: Gating strategies for platelet parameters in control and calcium ionophore A23187-activated platelets*